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# **TESTICULAR FUNCTION IN ADOLESCENT BOYS WITH KLINEFELTER SYNDROME**

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ACADEMIC DISSERTATION

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## ABSTRACT

Klinefelter syndrome (KS) is the most frequent karyotype disorder of male reproductive function. Since its original clinical description in 1942 and the identification of its chromosomal basis 47,XXY in 1959, the typical KS phenotype has become well recognized, but the mechanisms behind the testicular degeneration process have remained unrevealed. The current study was undertaken to increase knowledge about testicular function in adolescent KS boys. The purpose was to find targets for therapeutic interventions, especially considering androgen deficiency and infertility.

This prospective study comprised a longitudinal follow-up of growth, pubertal development, and serum reproductive hormone levels in 14 prepubertal and pubertal KS boys, aged 10.0 to 13.9 years at the start of the study. Each boy had a testicular biopsy, which was cryopreserved for possible future use in assisted reproductive techniques in adulthood. A small part of each biopsy specimen was thoroughly analyzed with histomorphometric and immunohistochemical methods. The impact of genetic features of the supernumerary X chromosome on growth, pubertal development, and testicular degeneration was also studied.

The KS boys had sufficient testosterone levels to allow normal onset and progression of puberty. Their serum testosterone levels remained within the low-normal range throughout puberty, but from midpuberty onwards, signs of a relative testosterone deficiency emerged. Findings like a leveling-off in testosterone and insulin-like factor 3 (INSL3) concentrations, high luteinizing hormone (LH) levels, and exaggerated responses to gonadotropin-releasing hormone (GnRH) stimulation suggest diminished testosterone secretion. But overall, no indisputable androgen deficiency appeared in the KS boys, and thus they would require no androgen supplementation before midpuberty.

A continuous increase occurred in serum INSL3 concentrations during pubertal development in the healthy boys. Hence, INSL3 may serve as a novel marker for onset and normal progression of puberty. Both in the healthy subjects and in the KS subjects, secretion of INSL3 was dependent on LH, and during pubertal development there existed a strong positive correlation with serum LH and testosterone levels as well as with testicular volume. In KS subjects, measurements of serum INSL3 may be useful in monitoring Leydig cell function.

In the KS boys the number of germ cells was already markedly lower at the onset of puberty. The pubertal activation of the pituitary-testicular axis accelerated

germ cell depletion. In KS, germ cell differentiation is—at least partly—blocked at the spermatogonium or early primary spermatocyte stages, since no pachytene spermatocytes were detected. In adolescent KS boys the presence of germ cells correlated with serum reproductive hormone levels.

In the KS testes, immature Sertoli cells were incapable of transforming to the adult type, and during puberty the degeneration of Sertoli cells increased markedly. Both inhibin B subunits were expressed in the Sertoli cells of KS subjects that had undetectable serum inhibin B concentrations, a phenomenon indicating an altered inhibin B synthesis in KS. The older KS boys displayed an evident Leydig cell hyperplasia, as well as fibrosis and hyalinization of the interstitium and peritubular connective tissue. Altered immunoexpression of the androgen receptor (AR) suggests that in KS boys during puberty a relative androgen deficiency develops at testicular level.

Genetic features of the X chromosome appear to play a part in modulating KS phenotypes. The present study suggested that parental origin of the supernumerary X chromosome and the length of the CAG repeat of the *AR* gene influence pubertal development and testicular degeneration, but do not influence growth before puberty or body composition during puberty. Thus in KS, androgens may play a role in the pathogenesis of the testicular degeneration.

The current study characterized by several means the testicular degeneration process in the testes of adolescent KS boys and confirmed that this process accelerates at the onset of puberty. Although serum reproductive hormone levels indicated no hypogonadism during early puberty, the histological analyses showed an already markedly reduced fertility potential in prepubertal KS boys. Genetic features of the X chromosome affect the KS phenotype. Further studies are, however, needed to reveal the mechanisms responsible for the testicular degeneration and the phenotypic differences in KS.

## LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by Roman numerals I to V:

- I. Wikström AM, Raivio T, Hadziselimovic F, Wikström S, Tuuri T, Dunkel L. Klinefelter syndrome in adolescence: onset of puberty is associated with accelerated germ cell depletion. *J Clin Endocrinol Metab* 89: 2263-2270 (2004)
- II. Wikström AM, Dunkel L, Wickman S, Norjavaara E, Ankarberg-Lindgren C, Raivio T. Are adolescent boys with Klinefelter syndrome androgen deficient? A longitudinal study of Finnish 47,XXY boys. *Pediatr Res* 59: 854-859 (2006)
- III. Wikström AM, Painter JN, Raivio T, Aittomäki K, Dunkel L. Genetic features of the X chromosome affect pubertal development and testicular degeneration in adolescent boys with Klinefelter syndrome. *Clin Endocrinol (Oxf)* 65: 92-97 (2006)
- IV. Wikström AM, Bay K, Hero M, Andersson AM, Dunkel L. Serum insulin-like factor 3 levels during puberty in healthy boys and boys with Klinefelter syndrome. *J Clin Endocrinol Metab* 91: 4705-4708 (2006)
- V. Wikström AM, Hoei-Hansen CE, Dunkel L, Rajpert-De Meyts E. Immunoexpression of androgen receptor and nine markers of maturation in the testes of adolescent boys with Klinefelter syndrome: evidence for degeneration of germ cells at the onset of meiosis. *J Clin Endocrinol Metab*, 92: 714-719 (2007)

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## ABBREVIATIONS

ABP	Androgen-binding protein
Ad	Spermatogonia of adult dark type
AMH	Anti-Müllerian hormone
Ap	Spermatogonia of adult pale type
AR	Androgen receptor
BA	Bone age
BMI	Body mass index
CA	Chronological age
CDP	Constitutional delay of puberty
CIS	Carcinoma <i>in situ</i>
CV	Coefficient of variation
ELISA	Enzyme-linked immunosorbent assay
E2	17 $\beta$ -estradiol
FSH	Follicle-stimulating hormone
G	Genital stage according to Tanner
GnRH	Gonadotropin-releasing hormone
hCG	Human chorionic gonadotropin
ICSI	Intracytoplasmic sperm injection
IHC	Immunohistochemistry
INSL3	Insulin-like factor 3
ISS	Idiopathic short stature
KS	Klinefelter syndrome
LGR8	Leucine rich repeat-containing G-protein receptor 8
LH	Luteinizing hormone
Lz	Letrozole
MAGE-A4	Melanoma antigen-encoding gene-A4
ns	Non-significant
<i>P</i>	Probability
P	Pubic hair stage according to Tanner
PI	Placebo
PSA	Prostate-specific antigen
RIA	Radioimmunoassay
S-	Serum

Sa	Sertoli cell of a type
Sb	Sertoli cell of b type
Sc	Sertoli cell of c type (adult type)
SCO	Sertoli cell-only
SHBG	Sex hormone-binding globulin
SD	Standard deviation
T	Testosterone
TESE	Testicular sperm extraction
TR-FIA	Time-resolved fluoroimmunoassay
Tvol	Testicular volume



## INTRODUCTION

Klinefelter syndrome was first described by Harry F. Klinefelter in 1942 as a clinical entity characterized by small firm testes, gynecomastia, hypogonadism, absent spermatogenesis, and higher than normal concentrations of follicle-stimulating hormone (FSH). This disorder was subsequently found to be caused by a supernumerary X chromosome (Jacobs and Strong 1959). Today, we know that about 80% of cases are due to the numerical chromosome aberration 47,XXY; the remaining 20% have higher-grade chromosome aneuploidies (48,XXXY; 48,XXYY; 49,XXXXY), 46,XY/47,XXY mosaicism, or structurally abnormal X chromosomes (Lanfranco et al. 2004). The phenotype of KS males progressively deviates from normal as the number of X chromosomes increases, whereas the KS subjects with mosaicism may have very few clinical symptoms (Lanfranco et al. 2004).

Based on chromosome surveys in newborn children the estimated prevalence of KS is about 1 in 600 newborn males, which makes KS the most common sex-chromosome abnormality (Bojesen et al. 2003). It is among the most frequent genetic causes of human infertility occurring in 11% of azoospermic men and in 4% of infertile men (Van Assche et al. 1996). Although the classical phenotype of KS is widely recognized, the disorder is severely underdiagnosed. A large Danish national registry study shows that only approximately one-fourth of adult males with KS are diagnosed (Bojesen et al. 2003). Less than one-tenth of the expected number are diagnosed before puberty (Abramsky and Chapple 1997; Bojesen et al. 2003).

As described by Klinefelter, the histology of the testes in the adult KS male is characterized by extensive fibrosis and hyalinization of the seminiferous tubules, and hyperplasia of interstitium (Klinefelter et al. 1942), but the tubules may show residual foci of spermatogenesis (Aksglaede et al. 2006). Introduction of testicular sperm extraction (TESE) in combination with intracytoplasmic sperm injection (ICSI) techniques has allowed nonmosaic KS males to father children (Schiff et al. 2005; Tachdjian et al. 2003). Testicular biopsies of prepubertal boys have shown preservation of seminiferous tubules with reduced number of germ cells, but Sertoli and Leydig cells have (when described) appeared to be normal (Ferguson-Smith 1959; Mikamo et al. 1968; Muller et al. 1995).

Until onset of puberty, 47,XXY boys have normal serum levels of testosterone, FSH, LH, and inhibin B; thereafter, however, during puberty, FSH and LH levels increase to hypergonadotropic levels, inhibin B decreases to undetectable levels, and

testosterone after—an initial increase—levels off at a low or low-normal level (Christiansen et al. 2003; Salbenblatt et al. 1985; Topper et al. 1982). Hence, most adult KS males display a clear hypergonadotropism with a varying degree of androgen deficiency; subsequently testosterone substitution therapy is widely used to avoid symptoms and sequels of androgen deficiency (Lanfranco et al. 2004).

To date, the mechanisms and pathogenesis behind the testicular degeneration process in KS are not known, and furthermore, the progression of this process has been incompletely described. Neither has any study revealed the mechanisms responsible for the wide variation in the 47,XXY phenotype, ranging from individuals with severe hypogonadism or behavioral problems in childhood or both to those with infertility as their sole presenting symptom as adults. This study was undertaken to characterize the testicular degeneration process in KS that most probably accelerates during puberty. The purpose was to find targets for therapeutic intervention with foci on infertility and androgen deficiency that could offer these patients a better prognosis and improvement in quality of life.

# REVIEW OF THE LITERATURE

## CLINICAL FEATURES OF KS

The following section describes the clinical features of KS according to age. Many of these findings may be attributed to the hypogonadism typical for this syndrome, but some are instead caused directly by the chromosome aberration.

### Neonatal period

KS is diagnosed prenatally by routine amniocentesis quite rarely, because the association with advanced maternal age is weak (Lanfranco et al. 2004; Simpson et al. 2003). Birth weight, length, and head circumference of KS infants have been reported as normal (Robinson et al. 1979; Ross et al. 2005) or low (Ratcliffe 1999; Stewart et al. 1979). Although most 47,XXY neonates appear normal at birth, the incidence of cryptorchidism and inguinal hernia (Ratcliffe 1982), and of minor anomalies (fifth-finger clinodactyly, high-arched palate) is increased (Ross et al. 2005; Visootsak et al. 2001).

### Childhood and adolescence

During childhood, the KS boy often presents with language delay, learning disabilities, or behavioral problems, and this often prompts child neurologists or child psychiatrists to perform chromosome analysis, along with fragile X screening (Abramsky and Chapple 1997; Visootsak et al. 2001). The mean intelligence of 47,XXY individuals is only slightly below that of controls, but a wide range of IQs has been noted (Ratcliffe 1999; Robinson et al. 1986; Simpson et al. 2003; Sorensen 1992; Stewart et al. 1986). In these studies, delayed speech development, difficulties in learning to read, decreased mathematical ability, and poor short- and long-term memory were common among the KS subjects. Furthermore, many had difficulties in relationships with peers and siblings; they were quiet and unassertive with diminished self-esteem. Neuromaturational delays include hypotonia, reduced gross and fine motor skills, coordination, speed, and strength (Robinson et al. 1986; Ross et al. 2005; Visootsak et al. 2001).

During childhood, a notable increase in height velocity occurs between 5 and 8 years of age, owing to greater leg growth, but the magnitude and timing of the pubertal growth spurt does not differ from that of normal boys (Ratcliffe 1999; Robinson et al. 1986; Stewart et al. 1986). A tendency to central obesity is common (Ratcliffe 1999).

Bone age demonstrates a pattern of slight early childhood delay, rising towards average values at age 7 to 8 (Stewart et al. 1986). Several studies have shown that onset of puberty is not delayed and that pubertal KS boys develop satisfactory secondary sexual characteristics (Ratcliffe 1999; Salbenblatt et al. 1985; Topper et al. 1982). The reported incidence of gynecomastia varies from 56% to 88% but in most is transient (Ratcliffe 1999; Salbenblatt et al. 1985; Visootsak et al. 2001).

Testicular volume of KS boys is already below normal during childhood (1.0-1.5 mL; normally 1.8 mL) (Laron and Hochman 1971; Ratcliffe 1982; Robinson et al. 1986). During puberty, testicular volume initially increases to about 4 mL (range 2-10) at midpuberty, but subsequently decreases to only 3 mL (range 2-7) during late puberty (Ratcliffe 1999; Salbenblatt et al. 1985; Topper et al. 1982). Normally, the estimated onset of release of spermatozoa (spermarche) occurs at a median age of 13.4 years, at Tanner stage P2-3, and between testicular volumes of 4.7 and 19.5 mL (Nielsen et al. 1986; Tanner 1962). Healthy boys may have their spermarche as early as at Tanner stage G2 (Hirsch et al. 1985).

## **Adulthood**

In adulthood the KS subjects are detected among patients with azoospermia presenting to infertility clinics (Abramsky and Chapple 1997). The adult KS male has a testicular volume of 4.0 to 5.5 mL (normal men at least 15 mL), and the testes are firmer than normal (Lanfranco et al. 2004; Smyth and Bremner 1998). Furthermore, the typical adult phenotype includes higher than normal height, abnormally long legs, and a feminine distribution of adipose tissue, including gynecomastia, decreased muscle mass, and sparse or absent facial, axillary, pubic, or body hair (Aks glaede et al. 2007; Lanfranco et al. 2004; Smyth and Bremner 1998). The KS patients often complain of decreased libido and potency, and muscle strength (Lanfranco et al. 2004). Bojesen et al. (2006) in a study based on hospital discharge diagnoses, report in KS subjects an increased morbidity in all but one of the main diagnostic groups. The highest hazard ratios were noted for congenital malformations, psychiatric disorders, and endocrine and metabolic disorders. These results are in agreement with numerous earlier results (Lanfranco et al. 2004; Simpson et al. 2003; Smyth and Bremner 1998). Concordantly, KS males have significantly increased mortality overall (Bojesen et al. 2004; Swerdlow et al. 2005a). They are at elevated risk for several cancers: lung cancer, breast cancer, non-Hodgkin lymphoma, and mediastinal tumors (Bojesen et al. 2006; Swerdlow et al. 2005b). Extragonadal germ cell tumors are associated with KS (Aguirre et al. 2006; Kaido et al. 2003).

Investigations of the neuroanatomic bases of the cognitive phenotype in KS have revealed a reduction in left temporal lobe gray matter, a finding consistent with the verbal and language deficits associated with KS (Giedd et al. 2006; Giedd et al. 2007; Itti et al. 2003; Patwardhan et al. 2000; Shen et al. 2004). The atrophy seems also to involve the limbic system, which agrees with findings of abnormalities of mood and behavior in XXY males (Giedd et al. 2006; Shen et al. 2004). Patwardhan et al. (2000) studied ten nonmosaic 47,XXY males diagnosed at birth, of which five had received testosterone supplementation therapy for 2 to 10 years from the mean age 17.4. These five treated KS subjects showed relative preservation of gray matter in the left temporal region and increased verbal fluency scores as compared with the five who went untreated (Patwardhan et al. 2000).

## **TESTICULAR FUNCTION IN KS**

The following section describes the age-specific data available on hormone levels reflecting testicular function and activity of the pituitary-gonadal axis in KS.

### **Fetal period**

When prenatal testosterone was investigated in amniotic fluid obtained at antenatal diagnosis between 16 and 20 weeks of gestation from 20 47,XXY fetuses and from 46,XY and 46,XX controls of the same age, no significant difference was noted between the two male groups; both had significantly higher testosterone levels than did the 46,XX fetuses (Ratcliffe et al. 1994).

### **Neonatal period**

At birth there already may exist some impairment of Leydig cell function. Cord-blood testosterone was significantly lower in two 47,XXY infants and in one 46,XY/47,XXY than in three control infants (Sorensen et al. 1981). However, another study comparing testosterone levels of six KS infants to levels in a large cohort of normal infants showed no significant difference (Ratcliffe 1982).

After birth, the pituitary-gonadal axis in healthy boys is strongly activated, which is manifested by pubertal or even adult levels of serum FSH, LH, testosterone, and inhibin B at the age of 3 months (Andersson et al. 1998a). After this minipuberty, hormone levels decline to normal prepubertal levels until pubertal reactivation of the pituitary-gonadal axis occurs. Lahlou et al. compared reproductive hormone levels

during minipuberty in 18 prenatally diagnosed 47,XXY boys to those in 215 healthy boys (Lahlou et al. 2004). The KS infants' timing of peak serum testosterone was similar to healthy infants', but the levels in the KS boys were significantly lower from birth to 8 months. In contrast, their serum LH, FSH, inhibin B, and anti-Müllerian hormone (AMH) levels were normal. Another study found in 11 of 12 KS boys under age 6 months lower than normal serum testosterone levels but normal gonadotropin levels (Ross et al. 2005).

## **Childhood and adolescence**

Prepubertal 47,XXY boys are characterized by normal serum levels of testosterone, FSH, LH, and inhibin B until onset of puberty (Christiansen et al. 2003; Salbenblatt et al. 1985; Stewart et al. 1986; Topper et al. 1982); their serum testosterone responses to human chorionic gonadotropin (hCG) stimulation are normal (Salbenblatt et al. 1985; Topper et al. 1982). During puberty, after an initial normal adolescent increase, serum testosterone concentrations plateau and subsequently remain within the low-normal range throughout puberty (Salbenblatt et al. 1985; Topper et al. 1982; Winter 1990). These testosterone levels seem sufficient to allow in pubertal KS boys development of satisfactory secondary sexual characteristics (Ratcliffe 1999; Salbenblatt et al. 1985; Topper et al. 1982).

Serum estradiol (E2) levels in early pubertal 47,XXY boys are high, and the E2 / testosterone ratios in particular remain high, irrespective of the presence or absence of gynecomastia (Gabrilove et al. 1979; Salbenblatt et al. 1985; Stewart et al. 1986).

From midpuberty (at about age 13) onwards, KS subjects show a gradual increase in FSH and LH concentrations to hypergonadotropic levels; FSH levels increase somewhat earlier and more markedly than do LH levels (Ratcliffe et al. 1986; Salbenblatt et al. 1985; Topper et al. 1982). At the same time, the responses of both FSH and LH to gonadotropin-releasing hormone (GnRH) stimulation become exaggerated (de Behar et al. 1975; Illig et al. 1975; Salbenblatt et al. 1985; Topper et al. 1982). The increasing LH concentration reflects a compensatory mechanism to maintain sufficient Leydig cell function. It has, however, been suggested that the exaggerated FSH secretion in particular may accelerate the primary process of fibrosis and hyalinization of the seminiferous tubules (Grumbach and Conte 1992; Salbenblatt et al. 1985; Topper et al. 1982).

Serum levels of inhibin B are considered to reflect Sertoli cell function during prepuberty and to become germ cell-dependent during midpuberty (Anawalt et al. 1996; Andersson et al. 1998b). Onset of normal male puberty is associated with increasing serum concentration of inhibin B, and by pubertal stage 2 the adult serum

level of serum inhibin B is already reached (Andersson et al. 1997; Raivio et al. 1998). In patients with KS, inhibin B similarly shows a progressive increase before the clinical onset of puberty, but this increase is followed by a decrease to low or unmeasurable levels (Christiansen et al. 2003; Lahlou et al. 2004). In healthy subjects, serum concentrations of AMH, another Sertoli cell marker, remain high throughout childhood and wane during normal male puberty concomitantly with rising testosterone levels and onset of meiosis in spermatogenesis (Al-Attar et al. 1997; Lee et al. 1996; Rey et al. 1993). This decrease in AMH levels most probably occurs also in KS subjects, since Lahlou et al. (2004) found unmeasurable AMH levels in six KS adolescents aged 14 to 18, although longitudinal data from pubertal KS boys is lacking.

## **Adulthood**

**Gonadotropins and sex steroids.** Adult KS patients are characterized by hypergonadotropic hypogonadism. Their concentrations of LH and FSH are high; FSH shows the best discrimination, with little overlap occurring with levels in normal individuals (Lanfranco et al. 2004; Smyth and Bremner 1998). Serum inhibin B levels in most adult KS subjects are undetectable (Anawalt et al. 1996; Christiansen et al. 2003; Klingmuller and Haidl 1997).

In 65 to 85% of adult KS patients, serum testosterone concentrations are below normal, but some show levels within the normal range (Aksglaede et al. 2007; Lanfranco et al. 2004; Smyth and Bremner 1998). Serum concentrations of sex hormone-binding globulin (SHBG) are high, causing a further decrease in biologically active free testosterone (Lanfranco et al. 2004). Since serum testosterone has a potent negative effect on leptin concentrations, the significantly higher serum leptin level in KS men than in normal males indicates diminished androgen activity at tissue level (Ozata et al. 1998; Wabitsch et al. 1997). On average, KS males have lower than normal E2 levels, but occasionally their serum E2 concentrations may be increased (Aksglaede et al. 2007; Jockenhovel 2004; Lanfranco et al. 2004). KS males have higher E2 / testosterone ratios than do normal males (Aksglaede et al. 2007).

**Insulin-like factor 3.** The peptide hormone INSL3, is a member of the relaxin-insulin family, is secreted by prenatal and fully differentiated adult Leydig cells (Ivell and Bathgate 2002), but is only weakly expressed in prepubertal Leydig cells and in Leydig cells that have become hypertrophic or de-differentiated (Ivell and Bathgate 2002; Klonisch et al. 1999). Measurements of INSL3 in normal men, in normal aging men, and in men with various testicular pathologies have shown that INSL3 reflects the functional status of the Leydig cells and that circulating INSL3 is entirely of testicular origin (Anand-Ivell et al. 2006a; Bay et al. 2005; Foresta et al. 2004). Recently, findings

reveal that adult KS patients have significantly lower than normal serum INSL3 concentrations (Bay et al. 2005; Foresta et al. 2004).

Production of both INSL3 and testosterone is related to LH (Bay et al. 2005; Bay et al. 2006; Foresta et al. 2004), but recent data strongly suggest that the production of these hormones is regulated differently; INSL3 secretion is probably dependent on the long-term trophic effect of LH on Leydig cell differentiation (Bay et al. 2005; Bay et al. 2006). INSL3 is essential for the transabdominal part of testis descent (Ivell and Bathgate 2002), but its biological significance in adults remains unclear. Serum INSL3 levels in adult males are relatively high, 0.5 to 1.7 ng/mL (Bay et al. 2005; Foresta et al. 2004), and the gene encoding the INSL3 receptor LGR8 (leucine-rich repeat-containing G-protein receptor 8) is expressed not only in the testis, but also in several other tissues such as the pituitary, thyroid, and kidney (Hsu et al. 2002). Kawamura et al. (2004) have suggested a paracrine role for INSL3 in germ cell survival through suppression of apoptosis. These findings suggest that INSL3 may play an important sex-linked endocrine role in the adult male.

## **TESTOSTERONE SUBSTITUTION THERAPY IN KS**

When testosterone serum concentrations in patients with KS become low, lifelong substitution therapy is indicated to prevent the symptoms and consequences of androgen deficiency, and subsequently to improve quality of life. Testosterone replacement results in increased masculinity, strength, libido, bone mineral density, and body hair. It has a positive effect on mood and behavior, and reduces fatigue (Lanfranco et al. 2004; Smyth and Bremner 1998). Beneficial effects of testosterone therapy in hypogonadal men have been demonstrated (Jockenhovel 2004; Lanfranco et al. 2004; Smyth and Bremner 1998), but it has, however, no positive effect on testicular size and fertility.

It has been proposed that adolescent boys with KS may benefit from early testosterone therapy; especially that androgen administration may have positive effects on cognition and behavior (Myhre et al. 1970; Nielsen et al. 1988; Patwardhan et al. 2000; Simpson et al. 2003; Sorensen 1992; Visootsak et al. 2001). Reports that verify hypoandrogenism in 47,XXY boys during puberty or examine their androgen status before start of treatment are, however, lacking. And, to date, no placebo-controlled studies showing the benefits of early testosterone substitution have been conducted.



Consequently, the optimal age for initiating androgen therapy remains to be determined.

## **TESTICULAR HISTOPATHOLOGY IN KS**

At present, whether the degeneration of seminiferous tubules in KS is due to a primary effect of the extra X chromosome on the development and function of germ, Sertoli, and/or Leydig cells, or whether it is due to defect interactions between these cells is unknown. Data are available on the age-specific changes in testicular histology in KS subjects, but in actuality, the natural history of testicular degeneration in KS has been incompletely characterized.

### **Fetal period**

As studies of aborted fetuses at gestational ages 18 to 22 weeks have clearly shown, the degenerative process may even start during fetal life (Coerdts et al. 1985; Murken et al. 1974). In the testicular biopsies of midterm 47,XXY fetuses, whereas the density and number of seminiferous tubules and mesenchymal structures appeared normal, a reduced number of germ cells and an increased proportion of tubules devoid of germ cells were visible (Coerdts et al. 1985). A testicular biopsy of a KS fetus aborted at 20 weeks of gestation with testes not yet descended showed pronounced hyperplasia of Leydig cells (Murken et al. 1974). However, similar histological changes can be seen in cryptorchid males with normal karyotypes (Hadziselimovic and Herzog 1990). In 47,XXY fetuses aborted at 17 and 20 weeks, two authors have reported normal testicular histology (Flannery et al. 1984; Gustavson et al. 1978)

### **Neonatal period**

Mikamo et al. (1968) showed, over the first year of life, a progressive diminution in the number of spermatogonia. That quantitative study of testicular biopsies of three 47,XXY infants aged 3, 4, and 12 months revealed a drop in their number of spermatogonia from 24 down to 0.1% of control value, but showed no effect on tubular diameter. The number and appearance of immature Sertoli cells appeared normal, as also did the interstitial tissue (Mikamo et al. 1968). A 13-day-old 47,XXY had germ cells in only 23% of his seminiferous tubules, with a reduced number of spermatogonia (Edlow et al. 1969). Numerous germ cells and immature Sertoli cells, plus normal Leydig cells were evident in a testicular biopsy of a 4-week-old KS infant undergoing

surgery for inguinal hernia, but a quantitative assay indicated a reduced number of spermatogonia (Ratcliffe 1982). The one-month-old 47,XXY infant in the group of KS boys studied by Muller et al. (1995) showed a normal number of germ cells in the biopsies despite bilateral undescended testes.

### **Childhood and adolescence**

The first report of a testicular biopsy in a chromatin-positive 10-year-old with KS showed normal histology (Bunge and Bradbury 1957). Based on this fact and on biopsies of two 11- and 13-year-old KS boys, Siebenmann and Prader made the interpretation that testes in KS subjects are normal until puberty, when development of the characteristic tubular hyalinization is preceded by depletion of germ cells (Siebenmann and Prader 1958). Ferguson-Smith, reporting in 1959 on eight retarded prepubertal chromatin-positive KS boys aged 7 to 12, noted the reduced size of their seminiferous tubules and a reduction in or complete absence of spermatogonia. A minority of the tubules were normal, containing a normal amount of spermatogonia; the majority were smaller tubules with undifferentiated Sertoli cells. The oldest patient, aged 12 and still prepubertal, showed maturation of Sertoli and Leydig cells but an almost complete absence of germ cells, and in addition, in some areas early tubular hyalinization. This boy also showed elevated urinary FSH secretion (Ferguson-Smith 1959).

Muller et al. (1995), studying testicular biopsies of 11 KS boys between the neonatal period and age 13, found no germ cells in their nine KS boys older than 2 years. The number of germ cells was normal only in a one-month-old 47,XXY infant and in a 4-year-old mosaic 46,XY/47,XXY boy. It should, however, be noted that in that study all were cryptorchid, a condition which also has a detrimental effect on the seminiferous epithelium (Hadziselimovic and Herzog 1990). In contrast, a 15-year-old KS boy with no history of cryptorchidism had sperm-containing tissue successfully extracted and cryopreserved, (Damani et al. 2001). Muller et al. (1995) did not perform qualitative or quantitative studies of Sertoli cells; Leydig cells found in a one-month-old and in a 13-year-old patient were judged as normal. Furthermore, mean tubular diameter was reduced in eight subjects, the exceptions being the three boys aged one month and 11 and 13 years; the two older boys showed signs of onset of puberty (Tanner stage P2) (Muller et al. 1995).

These hormonal and clinical data, and in addition, sparse histological observations, all indicate the possibility that when healthy boys are experiencing spermarche, KS boys' testicular function is at least in some respects relatively normal.

## Adult life

As described by Klinefelter in 1942, testicular histology in the adult patient is characterized by extensive fibrosis and hyalinization of the seminiferous tubules and hyperplasia of the interstitium. The patchy nature of testicular histology, with its more- and its less-affected areas, has been described (Foresta et al. 1999; Gordon et al. 1972; Heller and Nelson 1945; Nistal et al. 1982; Skakkebaek et al. 1969a; Skakkebaek 1969b; Steinberger et al. 1965). Foresta et al. (1999) for example, studied ten 47,XXY males aged 28 to 37 years and found the Sertoli-cell-only (SCO) pattern in eight of the ten biopsies, whereas two showed Sertoli cells and a few spermatogenic cells. Skakkebaek (1969b) when studying Sertoli-cell morphology, described two types of tubules: those containing either small immature Sertoli cells (chromatin positive) or containing larger and more differentiated Sertoli cells (chromatin negative). Later, studies of the cytologic features of such immature Sertoli cells have suggested a lower activity than that of mature Sertoli cells, probably resulting in compromised protein synthesis (Nistal et al. 1982). Furthermore, measurement of the amount of androgen-binding protein (ABP) secreted in fragments of seminiferous tubules *in vitro* has shown that KS men without testicular spermatozoa, when compared with KS men positive for spermatozoa, have a greater degree of Sertoli cell dysfunction (Yamamoto et al. 2002). Regadera et al. showed in their immunohistochemical and quantitative study that  $78.9 \pm 9.1\%$  of the Leydig cells were normal in the adult KS males compared to  $96.0 \pm 10.0\%$  in controls, and in KS that the functional activity of the Leydig cells was reduced (Regadera et al. 1991).

## Animal models

The development of XXY mouse models provides a tool to investigate maturation and degeneration of the KS testis (Bronson et al. 1995; Hunt and Eicher 1991; Lue et al. 2001). In the XXY testis during the period of germ cell proliferation in the early stages of testis differentiation, Hunt et al. (1998) noted significantly reduced germ cell numbers. They hypothesized that germ cell demise reflects a defect in somatic/germ cell communication, since in an *in vitro* system, the proliferative potential of fetal germ cells from XXY mice was indistinguishable from that of normal males. Furthermore, they noted a progressive decline in the number of germ cells during the early postnatal period until 12 days postpartum, when hardly any remaining germ cells were recognizable (Hunt et al. 1998). In the same manner, Lue et al. (2001) found that germ cells began to degenerate in the XXY mice at 7 days of age with a progressive loss resulting in a total absence of germ cells in the adult animals. Hence, germ cell loss in

XXY mice begins at a time when germ cells in normal mice reinitiate mitosis and before the onset of germ cell meiosis; hypertrophy and hyperplasia of Leydig cells and changes in Sertoli cells indicating cellular inactivity were also evident (Lue et al. 2001).

## **FERTILITY IN KS**

KS subjects are traditionally described as infertile, with only exceptional cases of spontaneous paternity reported (Laron et al. 1982; Terzoli et al. 1992). Semen analysis most often reveals azoospermia; in one cohort of KS males, only 8.4% had spermatozoa in their ejaculate (Lanfranco et al. 2004). Some spermatogonia in KS subjects are capable of completing the spermatogenic process leading to the formation of mature spermatozoa, but do so at an increased risk for genetic imbalance (Staessen et al. 2003). Two hypotheses have been proposed to explain this phenomenon: First, that 47,XXY spermatogonia undergo meiosis to produce hyperploid spermatozoa (Bielanska et al. 2000; Chevret et al. 1996; Ferlin et al. 2005; Foresta et al. 1999; Yamamoto et al. 2002); second, that rare patches of normal XY germ cells occurs, but as a result of a compromised testicular environment, these cells are susceptible to meiotic abnormalities (Bergere et al. 2002; Blanco et al. 2001; Mroz et al. 1998).

Natural conception thus rarely occurs for Klinefelter couples, and most often the only hope for biological paternity is testicular sperm extraction (TESE) combined with intracytoplasmic sperm injection (ICSI). The initial success rate of TESE in adult 47,XXY males in small series has been reported at 40 to 50% (Lanfranco et al. 2004); later, a rate has been achieved as high as 70% (Schiff et al. 2005). Once sperm have been obtained, live birth rates have been reported of 20 to 46% (Schiff et al. 2005; Staessen et al. 2003). The fact that spermatogenesis seems to decline with age makes it tempting to retrieve germ cells at an earlier age for cryopreservation and future utilization (Damani et al. 2001; Emre Bakircioglu et al. 2006; Ichioka et al. 2006; Lin et al. 2004; Okada et al. 2005). However, Schiff et al. (2005) could in their study not verify this age effect; they were able to perform several TESE procedures successfully for the majority of their patients.

In the KS male, the only predictive factor for successful sperm recovery seems to be testicular histopathology (Westlander et al. 2001), but even with no sperm found in a biopsy, TESE has been successful (Schiff et al. 2005; Vernaev et al. 2004). Neither testicular ultrasonography, extensive chromosome analyses, degree of virilization, testicular volume, nor serum testosterone, FSH, LH, nor inhibin B levels are

predictive for outcome of TESE (Vernaave et al. 2004; Westlander et al. 2001). In fact, even patients with inhibin B below the detection limit have undergone successful TESE (Westlander et al. 2003). To date, the births of more than 50 healthy children of KS fathers have been reported following the use of ICSI (Ferlin et al. 2005). In a triplet gestation, one 47,XXY fetus was reduced (Ron-El et al. 2000). Because of the risk of producing offspring with sex-chromosome abnormalities most investigators recommend professional genetic counseling and standard prenatal diagnosis techniques (Denschlag et al. 2004; Ferlin et al. 2005; Hennebicq et al. 2001; Staessen et al. 2003).

## GENETICS

For autosomal trisomies, maternal errors predominate, accounting for nearly 95% of cases. In contrast, the origin of 47,XXY is more complicated. Paternal meiosis I errors (leading to 47,X<sup>m</sup>X<sup>p</sup>Y) are common, accounting for 40 to 60% of cases (Iitsuka et al. 2001; Lanfranco et al. 2004; Simpson et al. 2003; Thomas and Hassold 2003). In the maternally derived cases (47,X<sup>m</sup>X<sup>m</sup>Y), the extra X chromosome is due either to non-disjunction during the first (MI) or second (MII) meiotic division, or in a post-zygotic mitotic division (Iitsuka et al. 2001; Lanfranco et al. 2004; Simpson et al. 2003; Thomas and Hassold 2003). No obvious increased risk for conception of an affected child occurs with advanced paternal age (Lanfranco et al. 2004; Simpson et al. 2003), but among maternally derived cases, an association with increasing maternal age exists, but this effect is limited to the subset of cases originating at MI (Thomas et al. 2001).

Several genetic features of the X chromosome have been proposed to affect the KS phenotype, but to date this issue has not been thoroughly investigated. The parental origin of the supernumerary X chromosome results in different doses of paternally and maternally derived genes. Furthermore, due to imprinting, the paternal and maternal alleles may be differentially expressed (Iitsuka et al. 2001). As a dosage-compensation mechanism in subjects with two X chromosomes, one of the X chromosomes is randomly inactivated (Iitsuka et al. 2001; Willard 2001). What has, however, been shown is that over 15% of X chromosomal genes escape inactivation (Carrel and Willard 2005), and consequently, a widely accepted hypothesis is that these genes are responsible for many of the features of KS (Simpson et al. 2003). As in normal females, skewed X chromosome inactivation, defined as > 80% preferential inactivation of one of the X chromosomes, can also occur in KS (Iitsuka et al. 2001; Willard 2001). This leads to predominant expression of the genes on one of the X

chromosomes, and in 47,X<sup>m</sup>X<sup>p</sup>Y subjects, skewed inactivation of the X<sup>m</sup> could result in a situation where only paternally derived genes are expressed. In the case of X<sup>p</sup> inactivation, the situation equals that of a normal male and this causes perhaps a less severe phenotype.

In cases of errors in MI or in mitosis, the 47,XXY subject has two identical X chromosomes (Lanfranco et al. 2004). This isodisomy can, as in cases of skewed X inactivation, lead to expression of recessive mutations of X-linked genes and, accordingly, to a more severe phenotype.

Androgen-related genes may play a particular role in modulating differences in KS phenotype. The androgen receptor (*AR*) gene is located on the X chromosome. The N-terminal domain of exon 1 of the *AR* gene contains a highly polymorphic CAG repeat, the length of which is inversely associated with receptor activity (Zitzmann and Nieschlag 2003). It is possible that a subtle modulation of AR function contributes to the variability in KS phenotypes, especially since most of these patients have low-normal or low androgen levels.

In the degeneration process of the testes in KS, inhibin B may be of special interest, since p120, a candidate for an inhibin B-binding protein, is encoded by the Xq25 (Chong et al. 2000). Furthermore, among genes on the X chromosome, a large number belong to the cancer-testis antigen family and are expressed in testicular germ cells (Scanlan et al. 2002; Wang et al. 2001). Mroz et al. showed that X-reactivation occurs during germ cell development in the XXY mouse, and it is assumed that for the survival of germ cells in the mature testis the proper X-chromosome dose is crucial (Mroz et al. 1999). Hence, molecular mechanisms induced by an altered dose of X-encoded genes in testicular cells may, during puberty, initiate the degeneration process in the testes of boys with KS.

## **AIMS OF THE STUDY**

The aims of this study were:

1. to determine whether adolescent KS boys start displaying signs of Leydig cell dysfunction during puberty by assessment of clinical and biochemical characteristics during a longitudinal observation period.
2. to characterize the testicular degeneration process in adolescent KS boys by histomorphometry and immunohistochemistry.
3. to investigate the impact of genetic features of the X chromosome on growth during childhood and adolescence, on onset and progression of puberty, and on the testicular degeneration process.

## **SUBJECTS AND METHODS**

### **ETHICS**

The parents of each boy gave their informed consent for their son's and their own participation in this study approved by the ethics committee for pediatrics, adolescent medicine and psychiatry of the Hospital District of Helsinki and Uusimaa.

### **SUBJECTS AND STUDY PROTOCOL**

From the pediatric endocrinology outpatient clinic at the Helsinki University Central Hospital, Hospital for Children and Adolescents, 14 subjects with the nonmosaic karyotype 47,XXY were enrolled. One boy, patient 14KS, was diagnosed by amniocentesis, and the other 13 boys were diagnosed between 5 and 10.5 years of age by child neurologists. Karyotype analyses were performed for nonendocrinological indications (problems with speech, learning, behavior). None of these patients had a history of previous cryptorchidism, nor were any on androgen therapy. Before the start of systematic prospective surveillance, these patients had been followed up irregularly at visit intervals of 6 to 24 months in the same clinic, and after this study, the routine clinical visits continued at an interval of 6 months. These visits included routine reproductive hormone analyses, physical examination with measurement of testicular size, and assessment of Tanner pubertal stage. Data from these clinical surveillance visits were collected from patient records and merged with data obtained during the prospective part of the study.

At the start of the systematic follow-up, the median age of the subjects was 11.5 years (range 10.0-13.9). They were followed prospectively for 4 to 25 months (median, 18). During the systematic surveillance, they visited the Hospital for Children and Adolescents every fourth month. At each visit, a physical examination including measurements of height, weight, and testicular volume, and staging of puberty was performed. Once a year, skeletal age was assessed and a GnRH stimulation test performed. Sera for hormone measurements were collected every fourth month or at least once a year. For genetic studies (III), blood samples were collected from all 14 boys and 27 parents. Reported heights of all parents were recorded.



From each KS subject (mean age 11.9; range 10.1-14.0 years) an open testicular biopsy specimen was taken under general anesthesia; three of the boys (patients 8KS-10KS) had a second biopsy specimen taken from the contralateral testis 1.0 to 1.6 years after the first one. The major portion of each of these 17 biopsy specimens was cryopreserved for possible further use in assisted reproduction in adulthood.

For comparison of serum testosterone and E2 levels in KS boys to values in healthy adolescent boys, recently published reference values came from 55 healthy Swedish boys (Ankarberg-Lindgren and Norjavaara 2004; Ankarberg-Lindgren 2005) (II). These boys, aged 5.0 to 18.6 years, had undergone serial sampling for 24-h serum testosterone and E2 profiles once or repeatedly during pubertal development; their venous blood samples were drawn six times during 24 h.

For comparison of longitudinal changes in serum concentrations of SHBG, PSA, leptin, FSH, LH, and inhibin B levels (II), values were used from 25 healthy, untreated Finnish boys with constitutional delay of puberty (CDP) previously followed up in the same unit of the Hospital for Children and Adolescents (Raivio et al. 2004; Wickman et al. 2001). For assessment of laboratory values for the CDP group when compared to the KS group, BA was used rather than chronological ages (CA), since BA in the CDP group was delayed. For assessment of laboratory values in KS boys CA was used (II), because differences between BA and CA in these boys during the study period were small.

In KS boys, to compare longitudinal changes during puberty in serum INSL3 levels and in simultaneously measured testosterone and LH levels (IV), values came from 30 healthy boys aged 9.0 to 14.5 years; they were previously enrolled in a study on idiopathic short stature (ISS) at the outpatient clinic for pediatric endocrinology of the Hospital for Children and Adolescents. The selection of patients and study protocol have been described in detail (Hero et al. 2005).

In order to study the effect of aromatase inhibitors on delaying bone maturation to increase predicted adult height, these ISS boys were randomized to receive either the aromatase inhibitor letrozole (Lz; Femar®, Novartis AG, Basel, Switzerland) at a dose of 2.5 mg or placebo (PI) orally once daily for 2 years. During treatment, these boys were examined every 6 months, and again 12 months after cessation of treatment. Their follow-up included the same investigations as the follow-up of the KS boys. BA rather than CA was used in the comparisons between the KS and ISS groups (IV), since the majority of the ISS boys had delayed BA.

## CLINICAL ASSESSMENT

Puberty was staged according to Tanner (Tanner 1962). The length and width of the testes were measured with a ruler to the nearest millimeter. Testicular volumes were calculated by the formula  $0.52 \times \text{width}^2 \times \text{length}$ , converted to milliliters (Hansen and With 1952) and expressed as the mean volume of the left and the right testis. Bone ages were determined by the method of Greulich and Pyle (Greulich and Pyle 1959).

Body mass index (BMI) was calculated as weight (kg) divided by height squared ( $\text{m}^2$ ) (II, III). Midparental height (SD) for each KS boy was calculated by subtracting 171 from the arithmetic mean of the parents' heights and dividing this difference by 10 (III). Predicting adult height for the KS subjects was by the method of Bayley and Pinneau (Bayley and Pinneau 1952) (III). Height in centimeters was converted to SD scores by means of age-specific growth norms for normal Finnish boys (Sorva et al. 1984) (III).

## LABORATORY ASSAYS

The blood samples were drawn between 0830 h and 1530 h. After clotting, the serum was separated by centrifugation and stored at  $-20^\circ\text{C}$  or  $-70^\circ\text{C}$  until analysis.

**GnRH stimulation test.** GnRH (Relefact®, 3.5  $\mu\text{g}/\text{kg}$ , maximum 100  $\mu\text{g}$ ; Hoechst Marion Roussel, Deutschland GmbH, Frankfurt, Germany) was administered intravenously. LH concentrations were measured from samples obtained at 0 (before), 20, 30, and 60 minutes, and FSH concentrations at 0, 30, 60, and 90 minutes after administration of GnRH. The GnRH-induced gonadotropin response was defined as the difference between the basal and the GnRH-induced peak gonadotropin concentrations.

**Gonadotropins.** Serum FSH and LH levels were measured by ultrasensitive immunofluorometric assays, as described (Dunkel et al. 1990). The sensitivity of the assays was 0.05 IU/L, but FSH and LH concentrations  $< 0.1$  IU/L were treated as 0.1 IU/L. For FSH, interassay CV was  $< 3.3\%$  and intraassay CV  $< 4.4\%$ ; for LH these were  $< 4.4\%$  and  $< 4.1\%$ .

**Testosterone.** Serum testosterone levels were measured by two different radioimmunoassays (RIA). The first assay was applied to measure testosterone concentrations (I, III, V) after separation of steroid fractions on a Lipidex-5000 microcolumn (Packard-Becker, B. V. Chemical Operations, Groningen, The

Netherlands) (Apter et al. 1976). The detection limit for testosterone was 0.1 nmol/L. The interassay CV was < 15% and the intraassay CV was < 9%. The second method (II, IV) was an ultrasensitive (sensitivity 0.03 nmol/L) modified RIA (Spectria testosterone, Orion Diagnostica, Espoo, Finland) (Ankarberg and Norjavaara 1999). The intraassay CV was 11% at 0.2 nmol/L, and < 7% at concentrations > 0.9nmol/L; the corresponding interassay CV was 15% and < 10%, respectively.

**17 $\beta$ -estradiol.** Serum E2 concentrations were measured with a modified RIA (Spectria estradiol, Orion Diagnostica) after diethyl ether extraction (Ankarberg-Lindgren 2005; Norjavaara et al. 1996). The assay detection limit was 4.5 pmol/L, the intraassay CV was 13% at 19 pmol/L, and interassay CV 12% at 22 pmol/L.

**Sex hormone-binding globulin.** Serum SHBG concentrations were measured by time-resolved fluoroimmunoassay (TR-FIA, Perkin-Elmer Life Sciences, Turku, Finland) with inter- and intraassay CVs both < 5% according to the manufacturer.

**Leptin.** Serum leptin was quantified with a commercially available RIA from Linco Research Inc., St. Charles, MO, USA. The detection limit of this assay is 0.5  $\mu$ g/L. Interassay CV was < 7% (concentration range 5-25  $\mu$ g/L).

**Prostate-specific antigen.** Serum PSA was quantified with a time-resolved immunofluorometric assay (Prostatus PSA EQM DELFIA, Wallac, Turku, Finland). The detection limit for PSA was 0.02  $\mu$ g/L. Interassay CV was < 4% at PSA concentrations 0.2 to 100  $\mu$ g/L.

**Inhibin B.** Serum inhibin B levels were measured by a commercially available immuno-enzymometric assay according to manufacturer's instructions (Serotec, Oxford, UK). The detection limit was 15.6 pg/mL. The interassay CV was < 15% and the intraassay CV < 5%.

**Anti-Müllerian hormone.** Serum AMH levels were measured by a commercially available immunoenzymometric assay according to manufacturer's instructions (Immunotech-Coulter, Marseille, France). The detection limit for AMH was 5.5. pmol/L, and the interassay CV was 13.4%.

**Insulin-like factor 3.** INSL3 serum concentrations were analyzed in a semicompetitive time-resolved fluorescence immunoassay (TR-FIA), based on purified synthetic hINSL3, an hINSL3-specific rat antiserum, an hINSL3 tracer labeled with chelated Eu<sup>3+</sup> ions, and a secondary goat anti-rat  $\gamma$ -globulin antibody immobilized on the surface of microtiter wells (Bay et al. 2005). This assay has a detection limit of 0.05 ng/mL and intra- and interassays CVs of 8.0 and 11.3%.

## ANALYSES OF THE TESTICULAR BIOPSIES

The major portion of each of the testicular biopsies of the 14 KS boys was cryopreserved for possible further use in assisted reproduction in adulthood.

**Histomorphometric analyses of the testicular biopsies (I).** For histomorphometric analyses, a piece of each testicular biopsy specimen was fixed in glutaraldehyde, then further subdivided and embedded in Epon, sectioned at 1.0  $\mu\text{m}$ , and stained with toluidine blue. Histomorphometric analysis was performed by light microscopy at a total magnification of  $\times 400$ .

The Leydig cells were morphologically classed as fetal, juvenile, or adult types by the following criteria: Leydig cells were regarded as fetal if they had an extrinsically located large nucleus with two or more nucleoli, as juvenile if they had an irregular nucleus and dark cytoplasm, and as adult if they were large cells with a round nucleus and cytoplasm containing crystalloid and lipid droplets.

The Sertoli cells were morphologically classed as Sa, Sb, and Sc types and were regarded as of Sa type if they were round with scant cytoplasm and had a round nucleus, and as Sb if they were oval and larger, with an irregular oval nucleus and cytoplasm with recognizable structures. They were classified as Sc or adult if they were large and had a nucleus displaying one or more deep invaginations (Hadziselimovic and Herzog 1990; Nistal et al. 1982; Seguchi and Hadziselimovic 1974).

For all specimens containing germ cells, germ cell counts (number of adult pale type spermatogonia per seminiferous tubule, Ap / tubule, and number of adult dark type spermatogonia, Ad / tubule) were calculated per cross-section of tubule. Spermatogonia were regarded as type A if they were of an irregular shape with a round nucleus. Furthermore, they were regarded as Ap type if the nucleus had one or two nucleoli and as Ad if the nucleus had one or two pale round areas (Hadziselimovic and Herzog 1990). All tubules from an average of nine sections (range, 6-12) per biopsy specimen were studied. The average number of tubules studied per biopsy specimen was 130 (range, 71-177). Germ cell numbers were quantitatively compared to those of available normal testicular biopsies. Eleven identically prepared testicular specimens from adolescent boys were analyzed (11 yr,  $n = 5$ ; 13 yr,  $n = 4$ ; 15 yr,  $n = 1$ ; and 19 yr,  $n = 1$ ). Indications for these biopsies had been scrotal pain ( $n = 5$ ), hydatid torsion ( $n = 3$ ), retractile testis ( $n = 1$ ), and paratesticular fibrosis ( $n = 1$ ); one specimen was taken postmortem (accidental death).

For electron microscopy, two Epon-embedded specimens were sectioned at 50 nm with a Reichert E ultra microtome (Reichert Jung, Vienna, Austria) and stained with uranyl acetate and lead citrate. Observations were made with a JEOLJEM 1200 EX transmission electron microscope (JEOL, Tokyo, Japan).

**Immunohistochemical analyses of the testicular biopsies (V).** A small piece of each testicular biopsy specimen was fixed in formalin and embedded in paraffin. Thirteen identically prepared testicular specimens from the tissue archive of Helsinki University Central Hospital served as controls for the immunohistochemical experiments. These biopsies had been performed as part of a routine clinical screening for lymphoblastic infiltration in boys (mean age 11.0; range 7.5-16.5 yr) previously treated for acute lymphoblastic leukemia/lymphoma. These specimens were judged by morphological inspection to be normal. As adult controls, biopsy specimens were obtained during treatment from the histologically normal contralateral testes of 18- to 39-year-old men diagnosed with testicular carcinoma *in situ* (CIS) or with a testicular germ cell tumor (affected tissues served as positive controls) at the University Hospital (Rigshospitalet) in Copenhagen, Denmark.

The protein expression profile / pattern was analyzed by IHC with several antibodies; see Table 1 for details. The staining was performed on 4- $\mu$ m thick sections by a standard indirect peroxidase method as reported for several of the antibodies (Andersson et al. 1998b; Aubry et al. 2001; Bartkova et al. 2001; Hoei-Hansen et al. 2004; Rajpert-De Meyts et al. 1999; Rajpert-De Meyts et al. 2004; Satie et al. 2002; Visfeldt et al. 1999). Briefly, most of the dewaxed and rehydrated sections were pre-treated in a microwave oven in a buffer. Subsequently, the sections were incubated with 1.5% H<sub>2</sub>O<sub>2</sub> to inhibit the endogenous peroxidase, followed by diluted non-immune goat serum to block unspecific binding sites. Incubation with the diluted primary antibody was overnight at 4°C. For the negative control, at least one section was incubated with a dilution buffer for each investigation. Subsequently, a secondary biotinylated goat anti-mouse link antibody was applied, followed by a horseradish peroxidase-streptavidin complex. Between all steps, the sections were thoroughly washed. The bound antibody was visualized with an aminoethyl carbazole substrate (all reagents from Zymed, San Francisco, CA, USA). Sections were lightly counterstained with Mayer's hematoxylin.

Two observers (A.W. and C.H-H) systematically scored the stainings. If any discrepancy arose, slides were re-evaluated by a third observer (E.R-D) to lead to a consensus. Staining intensity of the cells or tubules was assessed, with scores being ++, strong staining; +, intermediate staining;  $\pm$ , weak staining; 0, no specific staining

detected. The proportion of stained cells or tubules in each section was semi-quantitatively evaluated as a percentage of positive cells/tubules.

**Table 1.** Characteristics of antibodies applied in the immunohistochemical analyses of the testicular biopsies.

Antibody	Inclusion criteria	Pretreatment	Dilution	Details
AP-2gamma	Gonocyte marker	Urea buffer	1:30	SC-12762; Santa Cruz Biotechnology Inc., CA, USA
Androgen receptor (AR)	X-linked androgen-related Sertoli and Leydig cell marker	TEG buffer	1:40	MS-443-P; NeoMakers, Fremont, CA, USA
Anti-Müllerian hormone (AMH)	Androgen-related immature Sertoli cell marker	Urea buffer	1:150	Gift from Richard L. Cate, x NeoMakers, Fremont, CA, USA
CHK2	Spermatogonia marker	Citrate buffer	1:5000	Gift from J. Bartek, Danish Cancer Society, Copenhagen, DK
inhibin alpha-subunit	Sertoli and Leydig cell marker	Citrate buffer	1:10	R1alpha, see Andersson et. al. (1998a)
inhibin betaB-subunit	Sertoli and Leydig cell marker	Citrate buffer	1:6	MCA-1661; Serotec, Raleigh, NC, USA
MAGE-A4	X-linked spermatogonia marker	Citrate buffer	1:200	Gift from Giulio Spagnoli, Ludvig Inst. for Cancer Research, Lausanne, CH
MIC-2	X-linked Sertoli cell marker	TEG buffer	1:50	CD99, M3601; DakoCytomation, Glostrup, DK
NY-ESO-1	X-linked spermatogonia marker	None	1:50	Gift from Giulio Spagnoli, Ludvig Inst. for Cancer Research, Lausanne, CH
OCT-3/4	Gonocyte marker	Urea buffer	1:250	SC-5279; Santa Cruz Biotechnology Inc., CA, USA

Monoclonal antibodies, mouse

TEG buffer = TRIS 6.06g/5L, EGTA 0.95g/5L, pH= 9.0; Urea buffer = 5% carbamide, pH = 8.5; Citrate buffer = 10 mmol/L, pH = 6.0.

## GENETIC STUDIES

Genomic DNA was extracted from whole EDTA blood with the PUREGENE® DNA isolation kit (Gentra Systems, Minneapolis, MN, USA). Parental origin of the X chromosomes were determined by genotyping each boy and both parents (with the exception of one father) at 10 microsatellite loci: DXS6807, DXS989, DXS1068, DXS1003, DXS6800, DXS6797, DXS1001, DXS984, DXS1193 and DXS1073. The length of the CAGn repeat in exon 1 of the *AR* gene and the degree of X-chromosome inactivation were determined essentially as outlined in Suzuki et al. (Suzuki et al. 2001). The CAG alleles were sized initially by genotyping, and the lengths of the

repeats subsequently confirmed by sequencing all homozygous samples. The degree of skewing of X inactivation was estimated for all heterozygous samples according to equations outlined in Iitsuka et al. (2001), using the peak area values for each allele. All genotyping and sequence reactions were electrophoresed in an ABI 3700 (Applied Biosystems, Foster City, CA, USA) and analyzed with GeneMapper v3.7 (Applied Biosystems) and Sequencer v4.5 (Gene Codes Corporation, Ann Arbor, MI, USA), respectively.

X-weighted biallelic mean CAG repeat length was calculated by a method previously described (Hickey et al. 2002): Each allele in a genetic pair was multiplied by its percentage of expression (100% minus % inactivity) and summed.

## STATISTICS

Descriptive data are reported as medians and ranges or as means  $\pm$  SD. In those cases with no laboratory analyses at the time of testicular biopsy, values were interpolated from data obtained before and after the biopsy with the assumption that changes between the two time-points had been linear (I, V). To reduce frequency bias, means were calculated to obtain for each subject one value for each age or puberty stage (II, IV). Furthermore, because the same subjects were investigated repeatedly during follow-up, the method of summary measures was also used (Matthews et al. 1990) (II). Arithmetic means were first calculated for each individual; these individual means served as raw data in further tests. Spearman's rank correlations were calculated for associations between inhibin B and testosterone (I), AMH and testosterone (I), and between continuous parameters and X-weighted biallelic mean CAG repeat length (III). Differences between the Lz- and PI-treated groups of ISS boys were analyzed by repeated measures ANOVA (IV). Relations between INSL3 and other hormones were tested by linear regression analysis (IV). For comparisons between groups, the unpaired two-tailed Student's *t* test (I, II, IV) was used, and the non-parametric Mann-Whitney U-test (III, V) in the absence of normal distribution. A value of  $P < 0.05$  was considered statistically significant.

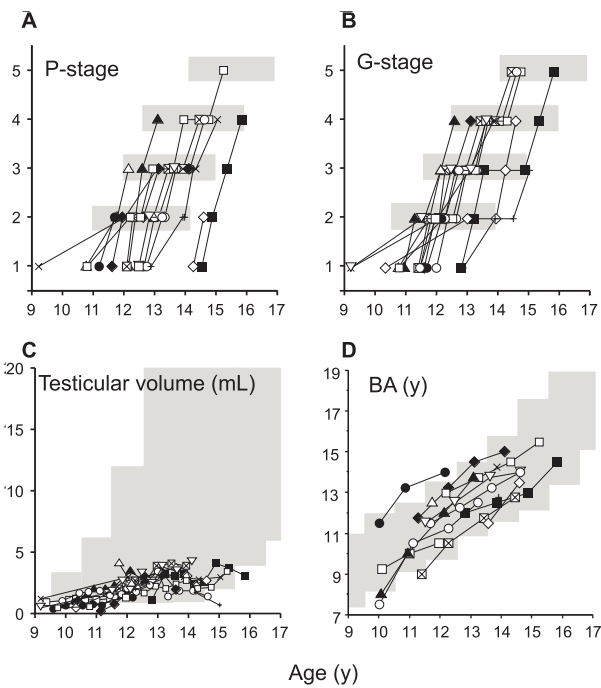
# RESULTS

## LONGITUDINAL OBSERVATION

### Clinical progression of puberty (II)

Figure 1 shows clinical progression of puberty in the 14 boys with KS. Both onset and progression of puberty, as assessed by Tanner stages, fell within the normal range for healthy Finnish boys (Ojajärvi 1982) (Figs. 1A and B, Table 2). At the end of the study period, only one (subject 1KS), aged 12.5 years, was at P1, but had entered puberty (stage G2). Although Tanner stages progressed normally during follow-up, there occurred an arrest in testicular growth, and testicular volumes of the 14 KS subjects never exceeded 4.5 mL (Fig. 1C and Table 2). Mean testicular volume 2.0 mL, accepted as indicating clinical onset of puberty, occurred at a median age of 12.6 years (range, 11.7-13.9,  $n = 13$ ), and 3.0 mL at 13.1 years (11.8-14.9,  $n=8$ ) (Fig. 1C and Table 2).

During follow-up, gynecomastia occurred in 11 KS boys; at G2 in two boys; at G3 in five, and at G4 in four. In five of these boys, it was reversible; when gynecomastia occurred, only one of these 11 boys had reached Tanner stage M3.



**Figure 1.** Pubertal development in 14 boys with KS. **A** and **B**: puberty staged according to Tanner. Gray areas = mean age  $\pm$  2 SD for healthy Finnish boys (Ojajärvi 1982). **C**: longitudinal changes in mean testicular volumes. Gray area = range of the volume of the right testis in healthy pubertal Swiss boys (Largo and Prader 1983). **D**: progression of bone age (BA) against chronological age. Gray area = mean BA  $\pm$  2SD for healthy Finnish boys (Ojajärvi 1982).



No significant delay in skeletal maturation during puberty occurred in the KS boys, as evidenced by a strong linear relationship between progression of bone age and chronological age (Fig. 1D). Only during prepuberty did three boys show a significant delay in bone maturation of 2.1 to 2.5 years, but it caught up with advancing age. One boy displayed an advanced BA of 2.4 years before onset of puberty. Otherwise, BAs of the KS boys fell within the mean  $\pm$  2 SD for healthy Finnish boys during puberty (Ojajärvi 1982) (Fig. 1D).

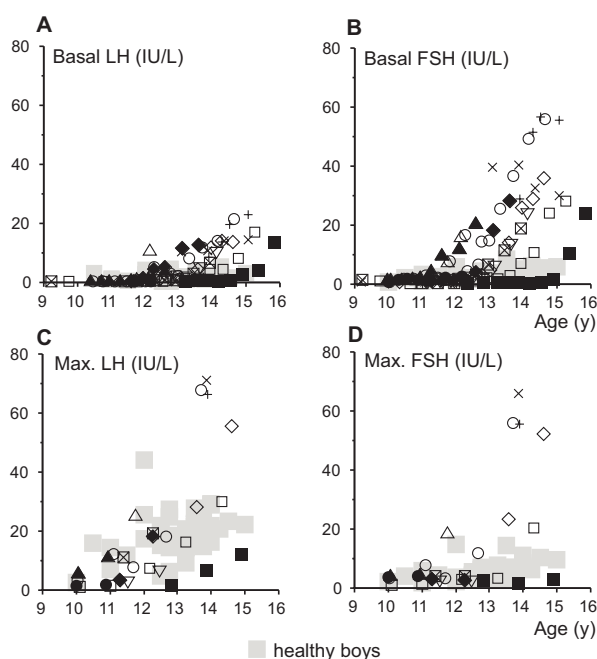
**Table 2.** Follow-up of 14 adolescent boys with KS. Means  $\pm$  SD (number of patients) and \*median, range (n) are displayed

Age (years)	10-11	11-12	12-13	13-14	14-15	15-16
Tanner P-stage*	1 (9)	1, 1-2 (9)	2, 1-3 (13)	2.5, 1-4 (11)	3, 1-4 (9)	4, 3-5 (4)
Tanner G-stage*	1 (9)	1, 1-2 (9)	2, 1-4 (13)	3.5, 2-4 (11)	4, 2-5 (9)	4, 3-5 (4)
Mean testicular volume (mL)	1.1 $\pm$ 0.5 (5)	1.8 $\pm$ 0.9 (9)	2.1 $\pm$ 0.5 (12)	2.9 $\pm$ 0.5 (11)	2.6 $\pm$ 0.7 (9)	2.4 $\pm$ 1.2 (4)
Bone age (years)	9.5 $\pm$ 2.1 (4)	11.0 $\pm$ 1.2 (7)	12.3 $\pm$ 1.1 (9)	13.1 $\pm$ 1.0 (11)	13.8 $\pm$ 0.8 (7)	15.0 $\pm$ 0.7 (2)
BMI (kg/m <sup>2</sup> )	17.7 $\pm$ 2.2 (8)	19.3 $\pm$ 2.7 (10)	19.3 $\pm$ 2.9 (13)	19.7 $\pm$ 2.4 (11)	19.7 $\pm$ 3.1 (9)	18.9 $\pm$ 2.0 (4)
Testosterone (nmol/L)	0.53 $\pm$ 0.43 (4)	0.78 $\pm$ 0.37 (9)	2.81 $\pm$ 2.68 (7)	5.65 $\pm$ 4.50 (7)	7.60 $\pm$ 3.62 (6)	
Estradiol (pmol/L)	10.9 $\pm$ 3.1 (3)	11.6 $\pm$ 5.5 (5)	16.4 $\pm$ 4.8 (6)	26.4 $\pm$ 16.5 (4)	32.5 $\pm$ 10.7 (4)	
SHBG nmol/L)	94 $\pm$ 46 (4)	80 $\pm$ 37 (8)	77 $\pm$ 44 (10)	52 $\pm$ 19 (11)	46 $\pm$ 13 (7)	31 $\pm$ 8 (2)
PSA ( $\mu$ g/L)	0.024 $\pm$ 0.022 (4)	0.038 $\pm$ 0.042 (9)	0.077 $\pm$ 0.058 (7)	0.216 $\pm$ 0.184 (7)	0.369 $\pm$ 0.254 (5)	
Leptin ( $\mu$ g/L)	14.1 $\pm$ 7.3 (4)	14.7 $\pm$ 7.9 (9)	14.7 $\pm$ 7.3 (11)	9.8 $\pm$ 6.0 (11)	8.9 $\pm$ 6.8 (9)	13.8 $\pm$ 8.0 (2)
Basal LH (IU/L)	0.2 $\pm$ 0.1 (6)	0.5 $\pm$ 0.3 (10)	2.3 $\pm$ 2.9 (13)	6.4 $\pm$ 3.5 (11)	11.4 $\pm$ 6.1 (9)	15.8 $\pm$ 5.8 (4)
$\Delta$ LH (IU/L) after GnRH stimulation (95% CI for boys with CDP)	3.6 $\pm$ 3.3 (4)	8.7 $\pm$ 7.9 (7) (5.8 - 14.2)	10.4 $\pm$ 6.6 (6) (12.8 - 23.2)	36.4 $\pm$ 24.4 (6) (14.4 - 20.4)	25.6 $\pm$ 16.2 (3) (16.2 - 25.4)	
Basal FSH (IU/L)	1.2 $\pm$ 0.6 (6)	2.6 $\pm$ 2.6 (10)	5.8 $\pm$ 6.1 (13)	19.8 $\pm$ 12.4 (11)	30.2 $\pm$ 16.8 (9)	32.8 $\pm$ 16.3 (4)
$\Delta$ FSH (IU/L) after GnRH stimulation (95% CI for boys with CDP)	2.3 $\pm$ 1.0 (4)	3.5 $\pm$ 3.5 (7) (0.5 - 2.9)	2.1 $\pm$ 1.5 (6) (1.5 - 3.9)	13.9 $\pm$ 11.7 (6) (1.7 - 3.3)	9.2 $\pm$ 7.5 (3) (2.7 - 5.1)	
Inhibin B (pg/mL)	90 $\pm$ 25 (4)	93 $\pm$ 64 (9)	96 $\pm$ 59 (12)	48 $\pm$ 52(11)	41 $\pm$ 60 (8)	23 (1)
AMH (pmol/L)	558 $\pm$ 151 (4)	817 $\pm$ 843 (9)	605 $\pm$ 342 (7)	238 $\pm$ 305 (6)	101 $\pm$ 125 (5)	
INSL3 (ng/mL)	< 0.05 (3)	0.12 $\pm$ 0.24 (7)	0.23 $\pm$ 0.23 (8)	0.57 $\pm$ 0.31 (9)	0.58 $\pm$ 0.08 (5)	0.88 (1)

To reduce frequency bias, means or medians were calculated for each subject to get one value for each age interval.  
CDP = constitutional delay of puberty.

## Changes in hormone levels (I, II, IV)

**Development of hypergonadotropism and exaggerated responses to GnRH.** To evaluate the activity of the pituitary-gonadal axis during puberty, both basal gonadotropin levels and the responses to GnRH stimulation were investigated. From midpuberty onwards, the 14 KS boys showed a gradual increase in FSH and LH concentrations to hypergonadotropic levels, FSH levels increasing somewhat earlier and more markedly than LH levels (Table 2 and Fig. 2). Moreover, the pathologically accentuated LH and FSH responses to GnRH stimulation developed during midpuberty, at ages of 13 to 14 years (Table 2 and Fig. 2).



**Figure 2.**

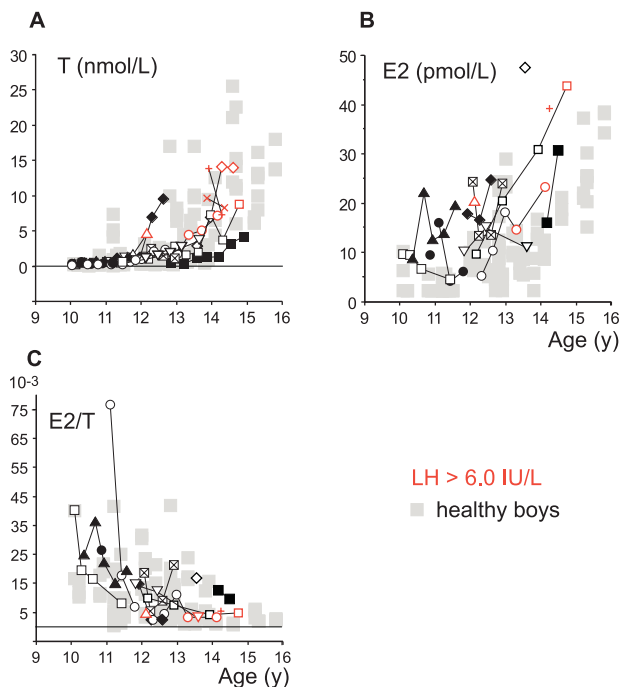
Development of hypergonadotropism in 14 boys with KS during puberty. Basal serum LH (**A**) and FSH (**B**) levels, and max. serum LH (**C**) and FSH (**D**) levels after GnRH stimulation, compared with values in healthy boys with constitutional delay of puberty (gray areas) (Raivio et al. 2004; Wickman et al. 2001). Chronological ages are shown for boys with KS and bone ages for boys with CDP.

**Serum testosterone levels during puberty.** Longitudinal changes in circulating testosterone levels in the KS boys and in 41 healthy boys of similar age (Ankarberg-Lindgren and Norjavaara 2004; Ankarberg-Lindgren 2005) are shown in Figure 3A. The diurnal rhythm of serum testosterone was taken into consideration by grouping the samples according to time of day; serum testosterone levels measured from 0747 to 1000 h or after 1000 h (1001-1510 h) were compared separately to those for the healthy controls measured at 0600 or 1400 h.

KS boys did not have lower serum testosterone levels than did controls in either group ( $P = ns$ ), but despite this, some of the older KS boys displayed hypergonadotropism, as evidenced by elevated ( $> 6$  IU/L) serum LH levels (Fig. 3A). In comparison with the group of 30 healthy boys with ISS, the KS boys displayed—at bone age 13 to 14 years and at Tanner stages G4 and P3, and concomitantly with pathologically high serum LH levels—significantly lower testosterone levels (Table 3); these, however, still fell within the normal testosterone range for healthy boys (Ankarberg-Lindgren and Norjavaara 2004).

**Longitudinal changes in serum estradiol and estradiol / testosterone ratios during puberty.** Especially before the age 12, the KS boys had higher serum E2 levels than did healthy controls (Ankarberg-Lindgren and Norjavaara 2004; Ankarberg-

Lindgren 2005). The E2 samples were also grouped according to time of day; differences in serum E2 levels were significant in both groups. Before 1000 h, in KS boys they were  $19.0 \pm 12.2$  pmol/L,  $n = 8$ , and in controls  $8.8 \pm 6.4$  pmol/L,  $n = 28$  ( $P = 0.003$ ). After 1000 h, in KS boys they were  $18.0 \pm 9.2$  pmol/L,  $n = 12$ , and in controls  $7.3 \pm 5.4$  pmol/L,  $n = 28$  ( $P < 0.0001$ ) (Fig. 3B). Differences in E2 / testosterone ratios (Fig. 3C) between the groups were not significant during prepuberty or puberty, but a tendency toward higher E2 / testosterone ratios appeared in the KS boys. Before 1000 h, in KS boys they were  $15.9 \pm 13.3$ ,  $n = 8$ ; in controls  $7.9 \pm 8.8$ ,  $n = 28$  ( $P = 0.05$ ). After 1000 h, in KS boys  $13.3 \pm 9.1$ ,  $n = 12$ ; in controls  $14.0 \pm 9.4$ ,  $n = 28$  ( $P = \text{ns}$ ). During follow-up, no tendency appeared toward higher E2 levels or higher E2 / testosterone ratios among the KS boys with gynecomastia than among those without (data not shown).

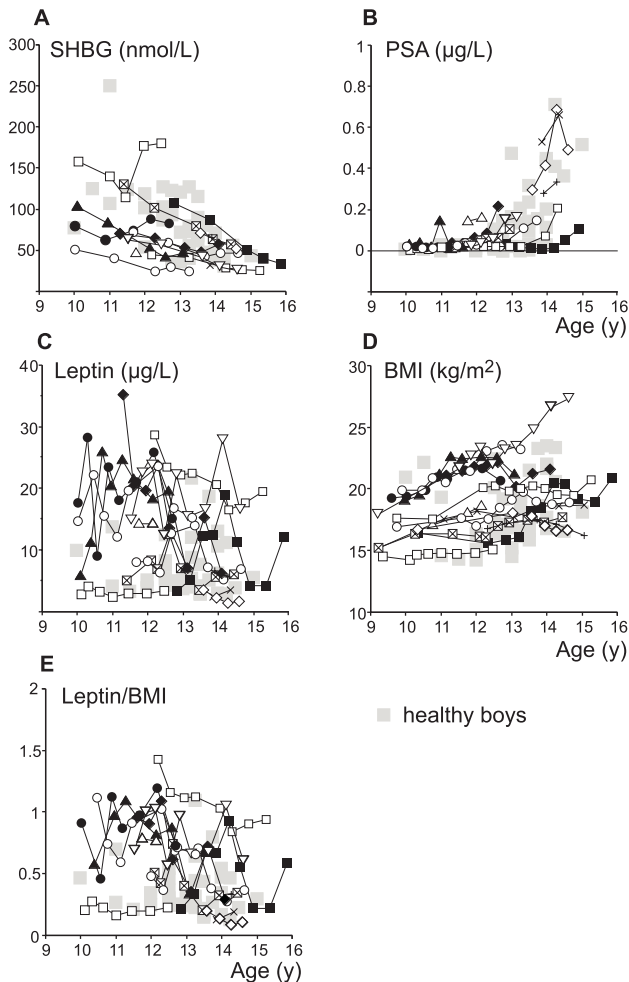


**Figure 3.**

Longitudinal changes during puberty in 14 boys with KS: **A**, serum testosterone (T) in KS subjects; **B**, serum 17β-estradiol (E2) in KS boys; **C**, E2 / T ratios in KS boys including those with (in red) and without serum LH > 6 IU/L. Levels in healthy Swedish boys (Ankarberg-Lindgren and Norjavaara 2004; Ankarberg-Lindgren 2005) were measured, at 0600 h, 1000 h, and 1400 h.

**Longitudinal changes in SHBG, PSA, and leptin during puberty.** To evaluate androgen activity at tissue level, serum SHBG, PSA, and leptin concentrations were measured. During puberty, serum SHBG levels in KS boys decreased normally, with levels remaining in the normal range ( $P = \text{ns}$ ) (Fig. 4A). Figure 4B shows longitudinal changes in serum PSA levels in KS boys; as compared to 25 healthy boys with CDP

(Raivio et al. 2004; Wickman et al. 2001), both timing and magnitude of rise in serum PSA in the KS boys were normal ( $P = ns$ ).



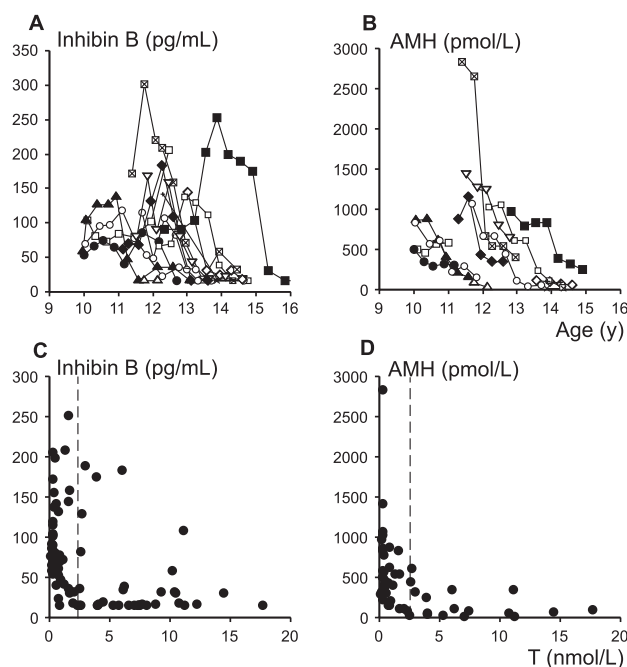
**Figure 4.**

Longitudinal changes in serum SHBG concentrations (A) prostate-specific antigen (PSA) (B), leptin (C), body mass index (BMI) (D), and leptin / BMI ratio (E) during puberty in 14 boys with KS, compared with levels in 25 healthy boys with constitutional delay of puberty (CDP) (Raivio et al. 2004; Wickman et al. 2001). Chronological ages are shown for boys with KS, and bone ages for boys with CDP.

Figure 4C shows longitudinal changes in serum leptin levels in the 14 boys with KS: Levels decreased during puberty, but were on average higher in the KS group than in healthy CDP controls (KS boys  $11.8 \pm 7.0$  μg/L; controls  $7.6 \pm 4.7$  μg/L,  $P = 0.03$ ; Fig. 4C). Mean BMI between these groups did not differ (KS boys  $19.0 \pm 2.4$  kg/m<sup>2</sup>; controls  $18.7 \pm 2.5$  kg/m<sup>2</sup>,  $P = ns$ , Fig. 4D). A slight increase in BMI during puberty appeared in both groups (Fig. 4D). When serum leptin levels were adjusted for BMI, leptin / BMI ratios declined with advancing puberty in both groups, but the KS boys still had higher ratios than did controls (KS boys  $0.57 \pm 0.30$ ; controls  $0.39 \pm 0.22$ ,  $P = 0.04$ , Fig. 4E).

**Longitudinal changes in inhibin B and AMH during puberty.** Serum inhibin B increased in early puberty, but this initial rise was followed by a rapid suppression accompanied by a simultaneous increase in serum testosterone (Fig. 5A and Table 2). A strong, inverse non-linear correlation ( $r_s = -0.57$ ,  $P < 0.0001$ ) existed between serum inhibin B and testosterone levels (Fig. 5C). When serum inhibin B levels declined, both basal FSH and responses to GnRH stimulation became clearly pathological (Table 2).

During prepuberty and early puberty, serum AMH levels were high, but with advancing puberty, AMH was suppressed simultaneously with inhibin B (Fig. 5B and Table 2). There also existed a strong, inverse non-linear relationship ( $r_s = -0.66$ ,  $P < 0.0001$ ) between serum AMH and testosterone levels (Fig. 5D). Before testosterone had reached 2.5 nmol/L, a marked drop in serum AMH had already occurred (Fig. 5D).



**Figure 5.**

14 boys with KS. **A** and **B**, longitudinal changes in serum inhibin B and serum anti-Müllerian hormone (AMH). **C** and **D**, correlations between inhibin B and testosterone (T), and AMH and testosterone. Dashed lines = serum testosterone 2.5 nmol/L.

**Longitudinal changes in INSL3 levels.** First, changes in serum INSL3 concentrations during puberty in healthy boys with ISS were characterized. In healthy boys, onset of puberty was associated with a marked increase in INSL3 levels (from Tanner pubertal stage 1 to 2) that occurred concurrently with significant increases in T and LH levels (Table 3). Significant increases in serum INSL3 and LH levels took place between bone ages 10 to 11 years and 11 to 12 years, and adult INSL3 concentrations  $\geq 0.55$  ng/mL (Bay et al. 2005) were reached at bone age 13 to 14 years (Table 3).

**Table 3.** Serum concentrations of INSL3, testosterone (T) and LH in 30 healthy boys with idiopathic short stature (ISS) at baseline, in 14 placebo-treated ISS boys during follow-up, and in 14 boys with Klinefelter syndrome (KS) according to bone age and Tanner pubertal stage.

	ISS boys			KS boys		
	INSL3 (ng/mL)	T (nmol/L)	LH (IU/L)	INSL3 (ng/mL)	T (nmol/L)	LH (IU/L)
Bone age (years)						
10-11	0.11 ± 0.12 (10)	1.2 ± 1.9 (10)	0.6 ± 0.6 (10)	0.00 (1)	0.9 ± 0.6 (4)	0.5 ± 0.3 (4)
11-12	0.30 ± 0.19 (10) *	2.7 ± 3.0 (9)	1.6 ± 0.8 (10) **	0.20 ± 0.24 (8)	1.2 ± 0.8 (7)	1.4 ± 1.7 (9)
12-13	0.46 ± 0.25 (7)	6.7 ± 3.7 (5) *	1.6 ± 0.8 (7)	<b>0.55 ± 0.30 (6) *</b>	4.0 ± 5.1 (8)	3.9 ± 4.3 (11)
13-14	<b>0.65 ± 0.14 (3)</b>	13.7 and 14.7 (2) §	3.2 ± 0.8 (3)	0.48 ± 0.40 (6)	5.3 ± 4.6 (7) §	6.5 ± 4.7 (9)
14-15	<b>0.66 ± 0.18 (3)</b>		3.0 ± 1.5 (3)	0.54 ± 0.27 (6)	5.4 and 9.6 (2)	9.9 ± 6.7 (7)
Pubertal stage G						
1	0.06 ± 0.05 (25)	0.4 ± 0.2 (25)	0.4 ± 1.5 (25)	0.03 ± 0.04 (6)	0.5 ± 0.3 (7)	0.2 ± 0.1 (7)
2	0.32 ± 0.16 (13) ****	2.1 ± 1.2 (11) ****	1.8 ± 0.9 (13) ****	0.28 ± 0.32 (11)	2.6 ± 3.0 (11)	2.5 ± 3.8 (13)
3	0.45 ± 0.15 (7)	7.5 ± 2.8 (6)	1.7 ± 0.8 (7)	0.47 ± 0.12 (6)	4.9 ± 4.5 (8)	5.1 ± 4.7 (11)
4	<b>0.78 ± 0.22 (6)</b>	15.1 ± 1.6 (3) §	0.7 ± 0.8 (6) §§	<b>0.59 ± 0.14 (5)</b>	6.3 ± 4.8 (6) §	8.5 ± 4.4 (9) §§
5	<b>0.56 and 0.81 (2)</b>		4.1 and 4.7 (2)	<b>0.68 ± 0.17 (4)</b>	8.8 (1)	14.1 ± 6.3 (4)
Pubertal stage P						
1	0.13 ± 0.14 (29)	1.1 ± 1.6 (29)	0.7 ± 0.6 (29)	0.11 ± 0.14 (9)	1.7 ± 2.5 (8)	1.1 ± 2.0 (9)
2	0.37 ± 0.16 (6) ***	3.9 ± 1.6 (4) **	1.7 ± 0.8 (6) **	0.45 ± 0.41 (5) *	5.2 ± 5.1 (9)	4.1 ± 4.3 (11)
3	<b>0.83 ± 0.23 (4)</b>	13.7 and 16.9 (2) §§	2.6 ± 0.8 (4) §	<b>0.59 ± 0.27 (8)</b>	4.8 ± 3.2 (7) §§	8.8 ± 5.5 (10) * §
4	<b>0.70 ± 0.16 (4)</b>	14.7 (1)	2.9 ± 1.3 (4)	<b>0.63 ± 0.18 (5)</b>	6.6 (1)	11.4 ± 6.9 (5)

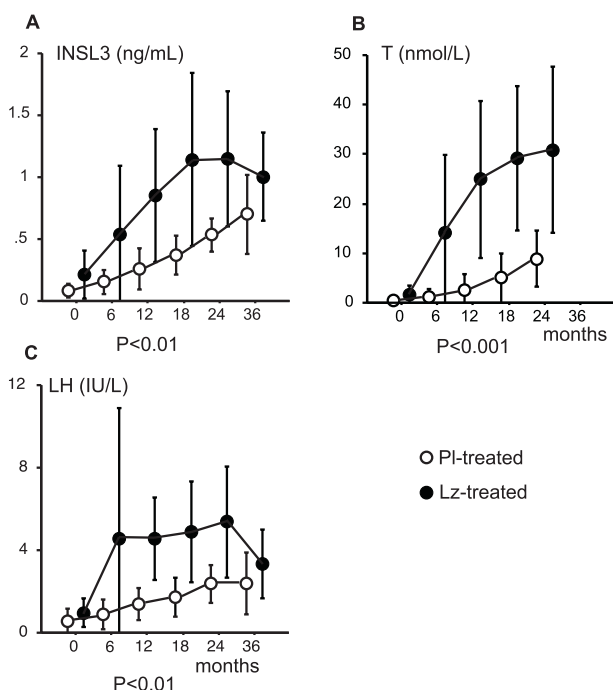
Means ± SD, number of patients (n). Bold indicates INSL3 levels > 0.55 ng/dL, 2.5 percentile in healthy adult men (Bay et. al. 2005)

\* and § indicate the stages when differences first became significant between stages within groups or between groups, respectively

\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  and \*\*\*\*,  $P < 0.0001$ . §,  $P < 0.05$  and §§,  $P < 0.01$

In KS boys in comparison with healthy boys, no significant difference in INSL3 levels emerged in assessment according to bone age or Tanner pubertal stage (Table 3). In the KS boys, the increase in serum INSL3 was significant between bone ages 11 to 12 and 12 to 13 years, and from Tanner pubertal stage P1 to P2 (Table 3). Adult INSL3 levels ( $\geq 0.55$  ng/mL) were reached at bone age 12 to 13 years. Thereafter, INSL3 and testosterone concentrations plateaued despite increasing LH levels (Table 3).

Following this, the impact of hypergonadotropism on serum INSL3 levels was analyzed during hyperandrogenism induced with an aromatase inhibitor in healthy boys with ISS. The 9 boys that entered puberty within 18 months from the start of Lz therapy showed a marked increase in serum INSL3 levels from  $0.21 \pm 0.19$  ng/mL (month 0) to  $1.15 \pm 0.55$  ng/mL (24 months), whereas the 8 PI-treated ISS boys, who entered puberty during follow-up, showed an increase from  $0.08 \pm 0.06$  ng/mL (month 0) to  $0.53 \pm 0.14$  ng/mL (24 months) (Fig. 6). One year after cessation of Lz therapy, a slight decrease in INSL3 levels was noted to  $1.00 \pm 0.36$  ng/mL (Fig. 6).



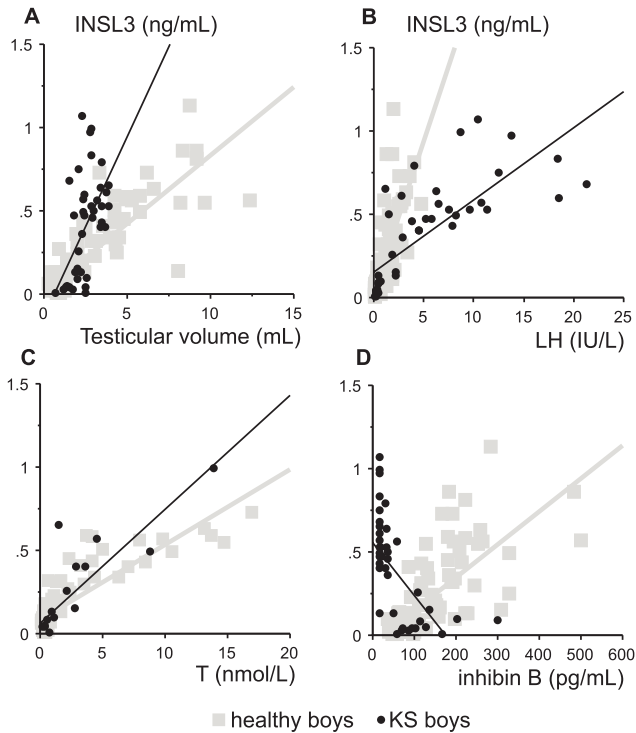
**Figure 6.**

Serum INSL3, LH, and testosterone (T) concentrations (mean  $\pm$  SD) in healthy boys with idiopathic short stature treated for 24 months with placebo (PI,  $n = 8$ ) or with letrozole (Lz,  $n = 9$ ).  $P$ -values, differences between the groups.

After this study, two of the KS boys (6KS and 7KS) were started on testosterone substitution therapy, and after 6 months on testosterone (Sustanon “250”®, Organon, Oss, Netherlands) 1 mg/kg every fourth week, their serum INSL3 decreased from 0.68 ng/mL to 0.11 ng/mL and from 0.49 ng/mL to 0.04 ng/mL. At the same time, their serum LH levels decreased from 21.3 IU/L to 4.1 IU/L and from 17.0 IU/L to 1.4 IU/L.

**Correlations of INSL3 levels during puberty.** Furthermore, study of relationships of INSL3 levels to testicular volume, serum LH, testosterone, and inhibin B levels showed that in the KS boys significant correlations appeared between INSL3 and testicular volume, LH, testosterone, and inhibin B concentrations, as they did also in both the PI- and Lz- treated ISS boys ( $|r| = 0.60-0.90$ ,  $P < 0.0001$ ). As testicular volumes in the KS boys never exceeded 4.5 mL, they displayed higher INSL3 / testicular volume ratios than did ISS boys ( $P < 0.0001$ ). The KS boys displayed low INSL3 levels when the hypergonadotropism with high LH levels occurred; this difference in INSL3 / LH ratios from those of the healthy boys was significant ( $P < 0.0001$ ). In INSL3 / testosterone correlations, the differences between these two groups were non-significant ( $P = 0.07$ ). Furthermore, the KS boys showed an inverse correlation between INSL3 and inhibin B

( $r = -0.63$ ,  $P < 0.0001$ ) because after onset of puberty secretion of inhibin B was very rapidly suppressed.



**Figure 7.** Healthy boys with ISS (placebo-treated) and boys with KS; in both groups significant ( $P < 0.0001$ ) correlations: **A**, between serum INSL3 levels and testicular volume; **B**, between INSL3 and LH; **C**, between INSL3 and testosterone (T), and **D**, between INSL3 and inhibin B levels. Linear regression lines are shown.



# MORPHOLOGICAL DEGENERATION OF THE TESTIS (I,V)

## Histomorphometric analyses

Results of histomorphometric analyses of the biopsy specimens, as well as the key characteristics of the 14 patients at the time of the first testicular biopsy are presented in Table 4. Representative specimens from five KS boys and from one boy with a normal karyotype are shown in Figure 8. The subjects were divided into two groups according to the presence (group I) or absence (group II) of germ cells from the biopsy specimens.

**Table 4.** Histomorphometric analyses of testicular biopsies of 14 boys with KS, with key characteristics at the time of the biopsy.

Patient number	Group number	Age (years)	Tanner stage	Mean testicular volume (mL)	Ap + Ad spermatogonia/ tubule	Ad spermatogonia/ tubule	Sertoli cells type	Leydig cell hyperplasia	Interstitial and peritubular connective tissue
1KS	I	10.1	P1/G1	1.1	1.2	0.01	Sa/Sb	0	fibr (+)
2KS	I	10.1	P1/G1	1.3	0.04	0	Sa/Sb	0	fibr +, hyalin
3KS	I	10.3	P1/G1	0.8	0.8	0.006	Sa/Sb	0	fibr (+)
4KS	I	10.7	P1/G1	1.6	1.0	0.03	Sa/Sb	+	fibr ++
5KS	I	11.6	P1/G1	1.0	0.1	0.01	Sa/Sb, pale	0	fibr +
6KS	I	11.9	P1/G1	1.8	1.2	0.01	Sa/Sb	0	fibr +, hyalin
7KS	I	12.5	P2/G2	1.7	1.2	0.03	Sa/Sb	0	fibr +, hyalin
9KS	I	11.9	P1/G2	2.5	+	+	Sa/Sb, degen	0	fibr +
10KS	I	13.0	P1/G2	1.8	+	+	Sa/Sb, degen	+	fibr ++, hyalin
12KS	I	13.7	P1/G2	3.4	+	+	pale, degen	+++	fibr ++
8KS	II	11.7	P1/G2	2.0	0	0	Sa/Sb	0	fibr (+)
11KS	II	11.8	P2/G2	3.9	0	0	Sa, pale, degen	++	fibr ++, hyalin
13KS	II	14.0	P2/G2	3.2	0	0	pale, degen	+++	fibr +++, hyalin
14KS	II	14.0	P3/G4	3.1	0	0	pale, degen, hyalin	++++	fibr +++, hyalin

+: presence of spermatogonia detected by MAGE-A4 staining (V), but not by morphological inspection (I); 0: no spermatogonia detected.

Patients are divided into two groups according to presence or absence of spermatogonia. Puberty staged according to Tanner. Ap (pale adult) and Ad (dark adult) spermatogonia counts per cross-section of seminiferous tubule. Sertoli cells staged as Sa, Sb, pale, and degenerating. The degree of Leydig cell hyperplasia staged 0 to +++. The degree of fibrosis of the interstitium is staged (+) to +++ and presence of hyalinization is marked "hyalin".

**Germ cells.** On morphological inspection, spermatogonia of the adult pale type (Ap) appeared in 7 of 14 and of the adult dark (Ad) type in 6 of 14 biopsy specimens (Table 4). The second biopsy specimens taken from patients 8KS to 10KS from the contralateral testis 1.0 to 1.6 years after the first specimens failed also to show any germ cells in histomorphometric analyses. In the immunohistochemical analyses, however, spermatogonia were detected by MAGE-A4 staining also in the samples of patients 9KS, 10KS, and 12KS (Tables 4 and 6). In the histomorphometric analyses, the average number of Ap spermatogonia per seminiferous tubule was reduced: mean number of Ap spermatogonia was 0.77 (range, 0.04-1.17), and average number of Ad

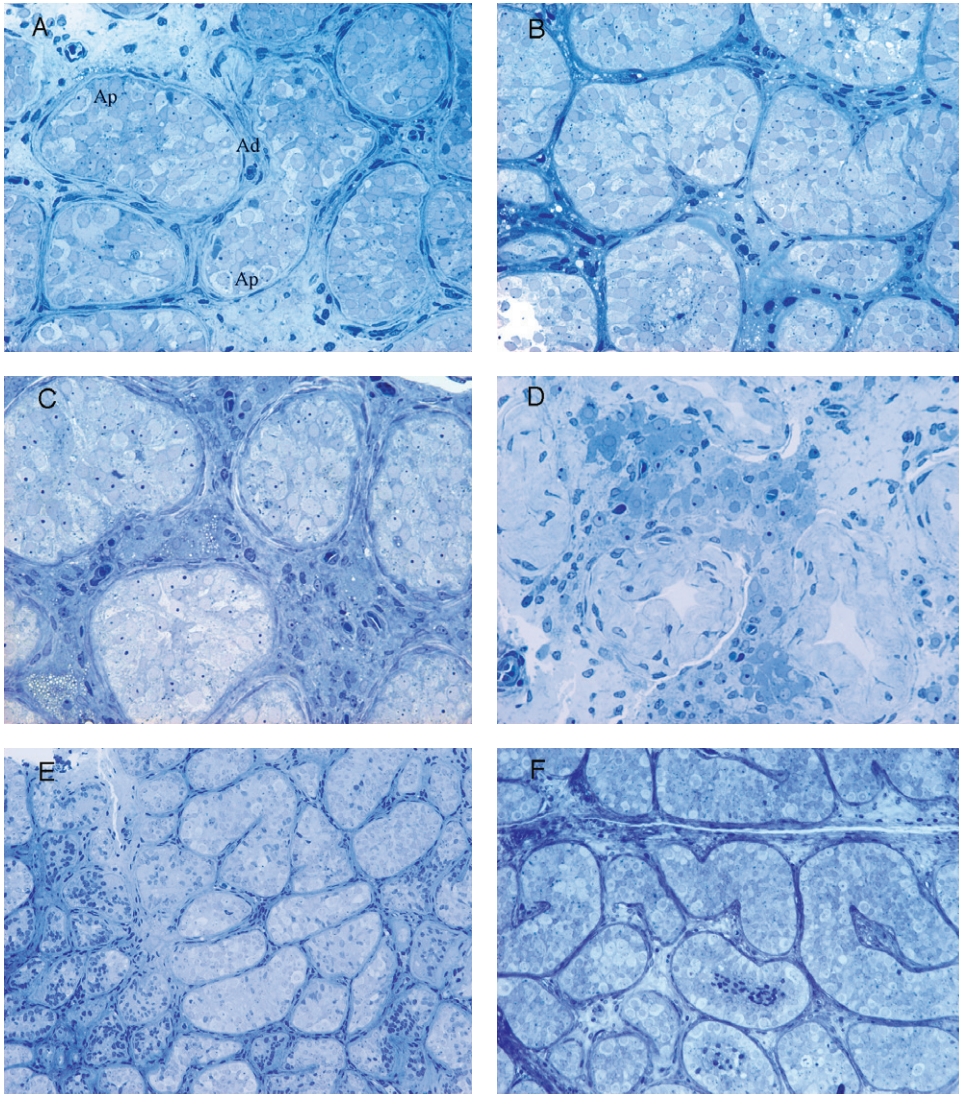
spermatogonia per tubule was 0.01, whereas all normal controls had consistently more than 0.46 (mean 1.5) Ad spermatogonia per tubule ( $P < 0.001$ ). No pachytene spermatocytes or post-meiotic spermatids appeared in any of the biopsy specimens of the KS subjects. Furthermore, at the time of cryopreservation of testicular tissue, no spermatozoa were visible in any of the specimens.

**Table 5.** Serum hormone concentrations of 14 boys with KS at the time of testicular biopsy.

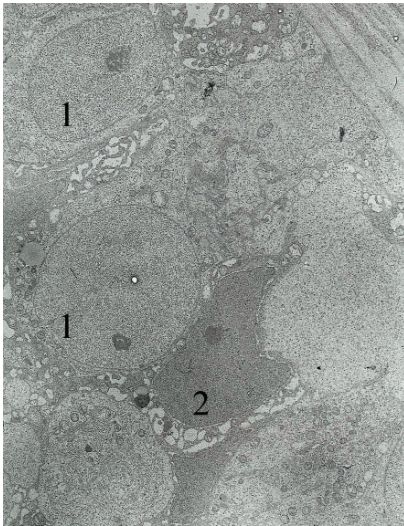
Patient number	Group number	FSH (IU/L)	LH (IU/L)	Testosterone (nmol/L)	inhibin B (pg/mL)	AMH (pmol/L)
1KS	I	0.1	0.1	0.3	103	503
2KS	I	1.0	0.2	0.3	78	758
3KS	I	1.5	0.1	0.3	66	345
4KS	I	1.9	0.4	0.3	127	606
5KS	I	1.0	0.3	0.8	68	1156
6KS	I	1.5	0.5	0.8	75	803
7KS	I	1.1	0.5	1.1	69	1062
9KS	I	0.5	0.2	0.3	166	1251
10KS	I	0.7	0.1	0.5	97	878
12KS	I	17.9	6.9	3.9	29	101
8KS	II	1.3	0.5	0.7	301	2658
11KS	II	7.5	1.2	2.3	<15.6	81
13KS	II	33.2	9.7	15.7	<15.6	96
14KS	II	38.6	11.4	10.2	<15.6	16

Patients are divided into two groups according to precense or absence of spermatogonia (see Table 4).

**Sertoli cells, Leydig cells and interstitial tissue.** In the biopsy specimens of patients 1KS to 10KS, the Sertoli cells were of Sa and Sb type and exhibited a relatively normal appearance (Table 4, and Figs. 8A and B, and 9). However, marked degeneration of Sertoli cells was evident in patients 11KS to 14KS (Table 4, Figs. 8C and D). Boys 1KS to 10KS had Leydig cells of juvenile type that showed none or only moderate hyperplasia, whereas the older subjects 11KS to 14KS had huge hyperplastic Leydig cells (Table 4, Figs. 8C and D). Fibrosis and hyalinization of the interstitium and peritubular connective tissue were visible in all groups; in addition, these signs of degeneration increased with age (Table 4). Figures 8 A to D show differing degrees of the degeneration process. This degeneration was not uniformly detectable throughout the relatively small biopsy specimens, whereas within the same biopsies, some areas showed marked degeneration and some areas only moderate changes (Fig. 8E).



**Figure 8.** Testicular biopsies of five adolescent boys with KS from Table 4, and one normal boy. Stain = Toluidine blue. **A.** Patient 4KS, two types of seminiferous tubules identifiable: those with no spermatogonia on the right and those with spermatogonia of adult pale type (Ap) on the left. "Ad" indicates spermatogonia of adult dark type. Sertoli cells: Sa and Sb type. Leydig cells: juvenile type, some hyperplasia. Interstitium: increased fibrosis. x 400. **B.** Patient 8KS, no spermatogonia. Sertoli cells: Sa and Sb type. Leydig cells: juvenile type. Interstitial compartment appears normal. x 400. **C.** Patient 12KS, no spermatogonia. Sertoli cells: Sa and Sb type, pale and degenerative. Interstitial compartment: increased volume, hyperplastic Leydig cells, and some fibrosis. x 400. **D.** Patient 14KS, seminiferous tubules completely hyalinized. Interstitial compartment: hyperplastic Leydig cells and extensive fibrosis. x 400. **E.** Patient 7KS, centrally seminiferous tubules with Ap or Ad spermatogonia or both. This area is surrounded by tubules in which the degeneration process is evident: tubules with pale degenerative Sertoli cells and small tubules with dark small apoptotic cells. x 200. **F.** Normal control, age 11 yrs, testicular biopsy taken at surgical exploration because of scrotal pain. x 200.



**Figure 9.**

Patient 2KS, group I (Table 4). Electron micrograph of testicular biopsy of boy with KS at age 10.1 yrs and Tanner stage P1G1. Undifferentiated Sertoli cells resembling Sa type with a round nucleus with a small nucleolus lacking lamellar body, and with crystalloids from Charcot-Bötscher (1). In the center, one degenerating Sertoli cell (2). x 2500.

## Immunohistochemical analyses

**Expression of markers for germ cell differentiation.** Expression of AP-2 $\gamma$  (TFAP2C, transcription factor activator protein-2) and OCT-3/4 (also known as POU5F1, a POU-family transcription factor) was analyzed in the seven youngest KS patients (1KS-7KS) and in the youngest controls (1C and 2C). None of the markers showed any expression in these sections, whereas the positive control sections of testicular CIS were, as expected, strongly positive.

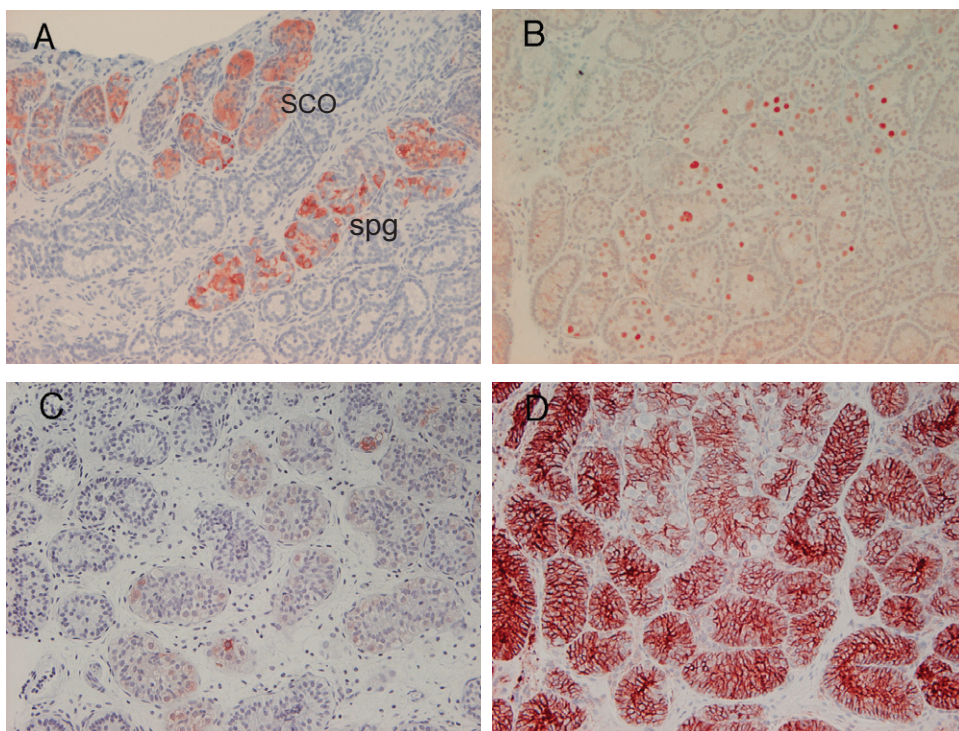
Then three established markers of spermatogonia: MAGE-A4 (Melanoma antigen-encoding gene-A4), NY-ESO-1 (CTAG1B/LAGE), and CHK2 (Chek2) (Table 6) were analyzed. MAGE-A4 was expressed in all spermatogonia in both KS and control specimens (Table 6 and Fig. 10A). In contrast, only a subset of spermatogonia was positive for CHK2 (40-80% of spermatogonia positive) and NY-ESO-1 (0-50% positive) (Table 6 and Figs. 10B and C). Spermatogonia were detected by MAGE-A4 staining even in samples (patients 9KS, 10KS, and 12KS) which by morphological inspection were judged to be negative for germ cells (I). Furthermore, some tubules with no apparent germ cells showed nonspecific expression of MAGE-A4 (Fig. 10A). No differences between KS and control samples were evident in intensity of expression of the X-linked markers MAGE-A4, NY-ESO-1, and MIC2 (Table 6 and Fig. 10)

**Table 6.** Immunoexpression of markers for spermatogonia (spg) and Sertoli cells in testicular biopsies of 14 prepubertal and pubertal boys with KS and in age-matched control boys (C)

Patient	Age (years)	Markers		MAGE-A4	%	NY-ESO-1		MIC-2
		CHK2	%				%	
1KS	10.1	++ / ±	50%	++	100%	±	30-50%	0
2KS	10.1	++ / ±	50%	++	100%	±	30-50%	0
3KS	10.3	++ / ±	50%	++	100%	±	30-50%	0
4KS	10.7	++ / ±	70%	++	100%	±	30-50%	0
5KS	11.6	++ / ±	70%	++	100%	±	30-50%	0
6KS	11.9	+ / ±	70%	++ / +	100%	±	30-50%	0
7KS	12.5	++ / ±	50%	++	100%	±	30-50%	no spg
8KS	11.7	n.a.		no spg		no spg		no spg
8KS	12.8	no spg		no spg		no spg		no spg
9KS	11.9	n.a.		+, few	100%	0	0%	0
9KS	12.9	no spg		++, few	100%	0	0%	no spg
10KS	13.0	n.a.		+, few	100%	0	0%	0
10KS	14.6	++ / ±		++, few	100%	±	30-50%	0
11KS	11.8	n.a.		no spg		no spg		no spg
12KS	13.7	no spg		++, few	100%	0	0%	no spg
13KS	14.0	n.a.		no spg		no spg		no spg
14KS	14.0	no spg		no spg		no spg		no spg
1C	7.5	++ / ±	80%	++	100%	0	0%	0
2C	7.5	+ / ±	70%	++ / +	100%	±	30-50%	0
3C	7.5	++ / ±	80%	++	100%	+ / ±	30-50%	0
4C	8.5	n.a.		n.a.		n.a.		0
5C	9.5	+ / ±	80%	++	100%	0	0%	0
6C	9.5	++ / ±	80%	++	100%	0	0%	0
7C	11.0	n.a.		n.a.		n.a.		0
8C	11.0	+ / ±	50%	++	100%	+ / ±	30-50%	0
9C	11.5	+ / ±	70%	++	100%	+	one spg	0
10C	12.0	+ / ±	few	++ / +	100%	no spg		0
11C	13.0	n.a.		n.a.		n.a.		0
12C	15.5	n.a.		n.a.		n.a.		0
13C	16.5	+ / ±	40%	++ / +	100%	±	30-50%	0
Adult normal testis		++ / ±	50-100%	++	100%	+ / ±	50-70%	0

Score: ++; strong staining, +; intermediate staining, ±; weak staining, 0; no specific staining detected  
%, approx. % positive spg detected in the section; n.a., not analyzed





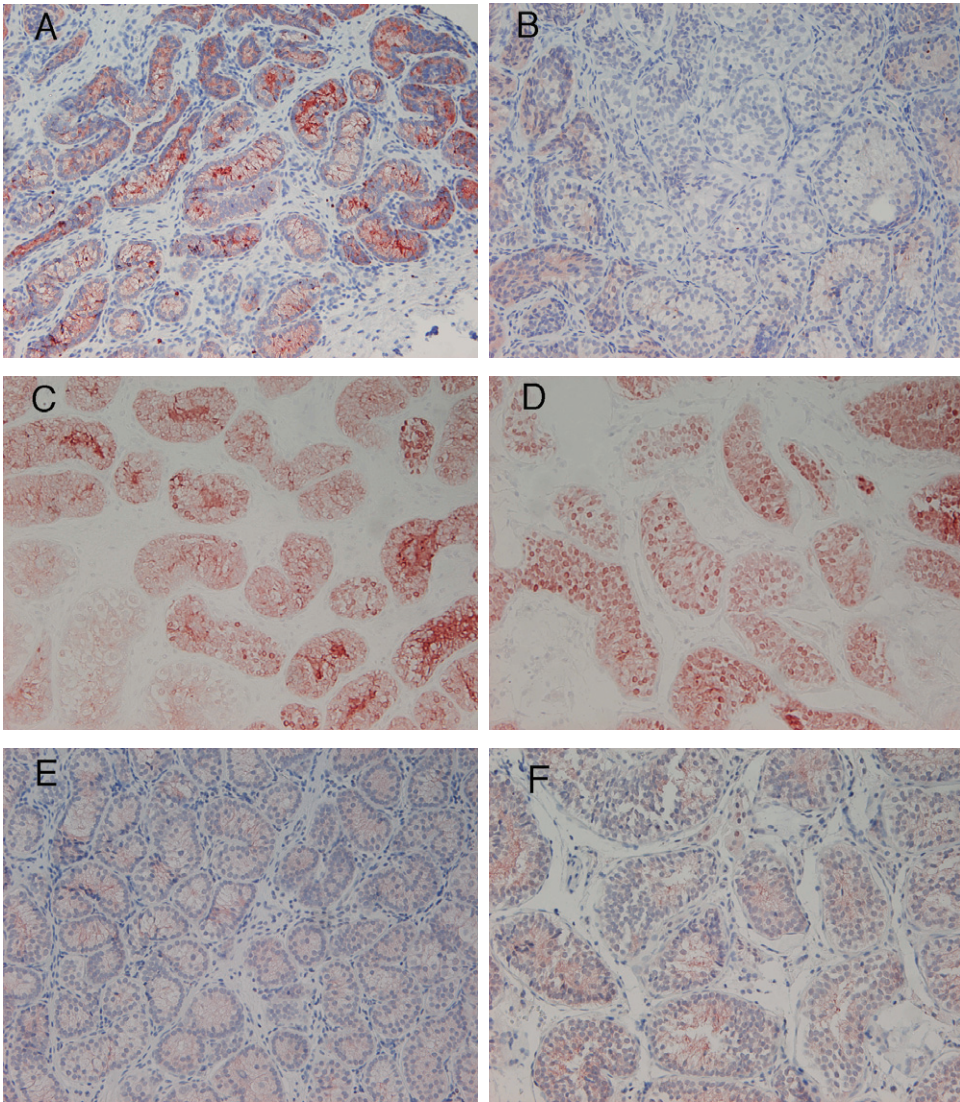
**Figure 10.** Markers for spermatogonia in testicular biopsies of boys with KS. See Table 6 for details. **A**, MAGE-A4 expression: seminiferous tubules with strongly stained spermatogonia (spg) and SCO tubules with unspecific or no staining (patient 1KS). **B**, CHK-2 expression (patient 4KS). **C**, NY-ESO-1 expression (patient 7KS). **D**, MIC2 expression: strongly stained Sertoli cells showing spermatogonia as white spots (patient 5KS). Magnification x 200.

**Expression of AMH.** Down-regulation with age of AMH expression in Sertoli cells occurred both in KS and in controls, but this event occurred at an older age in KS (Table 7 and Figs. 11A and B). A more heterogeneous picture appeared in both groups with decreased expression, with stronger staining in some tubules, whereas others were negative (Fig. 11B).

**Table 7.** Immunoeexpression of markers for Sertoli and Leydig cells in testicular biopsies of 14 prepubertal and pubertal boys with KS and of age-matched controls (C)

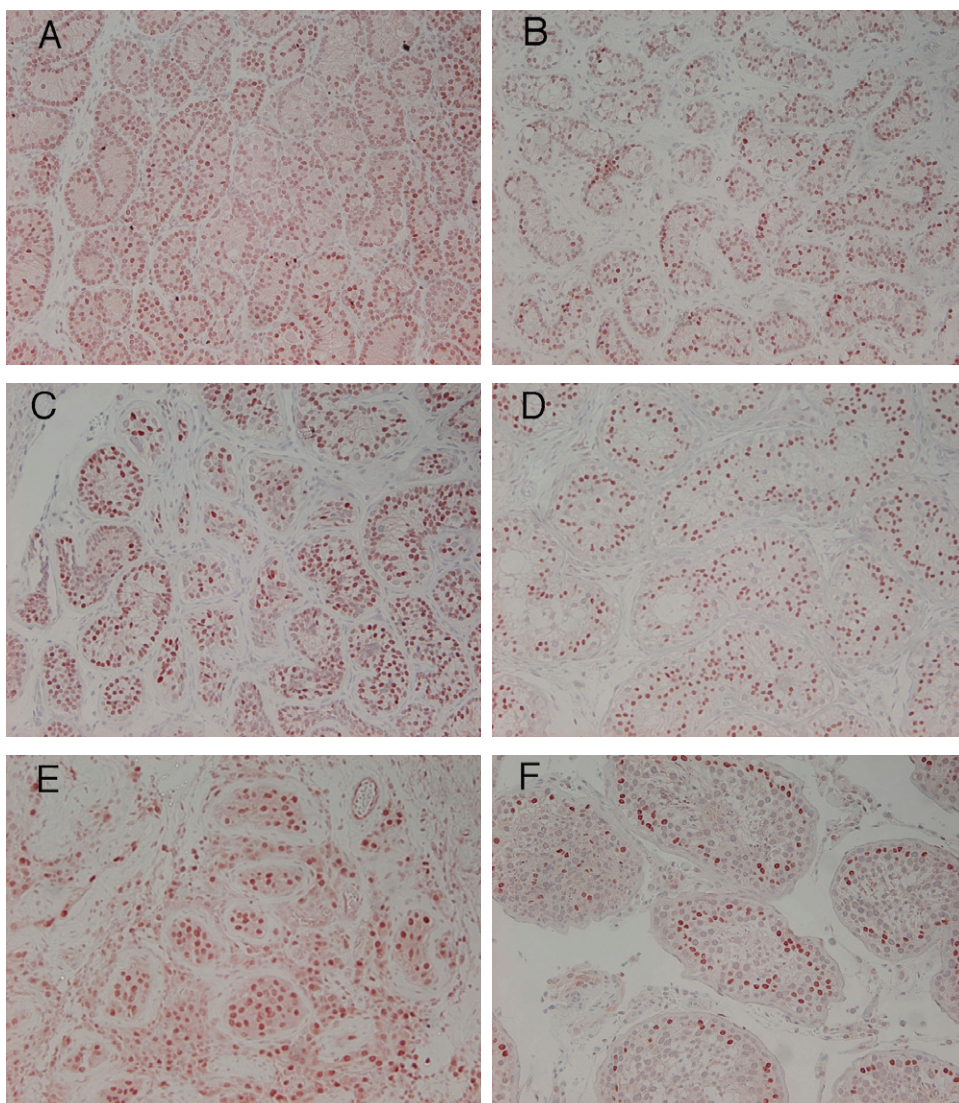
Patient	Age (years)	Markers AMH Sertoli cells	Inhibin alpha-subunit		Inhibin betaB-subunit		Androgen receptor (AR) Sertoli cells	Leydig cells*
			Sertoli cells	Leydig cells*	Sertoli cells	Leydig cells*		
1KS	10.1	++	++	0	+	0	N: ++ / ± (0-75%), C: ± / 0	N: 0, C: 0
2KS	10.1	++ / ±	++ / ±	0	±	0	N: ++ / + (10-75%), C: ± / 0	N: ± / 0 (33%), C: 0
3KS	10.3	++ / +	++	0	+	0	N: ++ / ± (10-75%), C: ± / 0	N: ± / 0, C: 0
4KS	10.7	+ / ±	++, N+	0	±	0	N: ++ / ± (33-100%), C: ± / 0	N: ± / 0, C: 0
5KS	11.6	++ / +	++	±	+	0	N: ++ / ± (10-75%), C: ± / 0	N: ± / 0, C: 0
6KS	11.9	+	++, N+	±	±	0	N: ++ / ± (10-50%), C: ± / 0	N: ±, C: 0
7KS	12.5	++ / +	++ / ±	0	±	0	N: ++ / ± (10-100%), C: ± / 0	N: ± / 0, C: 0
8KS	11.7	+ / ±	++ / ±, N+	±	+	0	N: ++ / ± (10-75%), C: ± / 0	N: ± / 0, C: 0
8KS	12.8	±	+ / ±, N+	0	±	0	N: ++ / ± (20-100%), C: ± / 0	N: ± / 0 (50%), C: 0
9KS	11.9	++ / ±	++ / +, N+	0	+	0	N: ++ / ± (10-75%), C: ± / 0	N: ± / 0, C: 0
9KS	12.9	±	+, N+	0	+	±	N: ++ / ± (40-100%), C: ± / 0	N: + / 0 (20%), C: 0
10KS	13.0	+ / ±	+	0	±	0	N: ++ / ± (5-50%), C: ± / 0	N: ± / 0, C: 0
10KS	14.6	+ / ±	++ / +, N+	±	+	+	N: + / ± (5-60%), C: ± / 0	N: ± / 0 (20%), C: 0
11KS	11.8	0	±, N+	±	+	±	N: ++ / + (80-100%), C: ± / 0	N: + / ± (60%), C: ±
12KS	13.7	+ / ±	+, N+	±	+	±	N: ++ / + (80-100%), C: ± / 0	N: + / ±, C: ±
13KS	14.0	trace	+, N+	±	+	0	N: ++ / ± (80-100%), C: ± / 0	N: + / ±, C: ±
14KS	14.0	0	±	++	+	+	N: ++ / + (90-100%), C: ± / 0	N: ++ / + (60%), C: ++ / ±
1C	7.5	+ / ±	+ / ±	0	±	0	N: ++ / ± (10-75%), C: ± / 0	N: ± (20%), C: 0
2C	7.5	+ / ±	++	0	±	±	N: ++ / ± (5-80%), C: ± / 0	N: ± (30%), C: 0
3C	7.5	+ / ±	n.a.	n.a.	n.a.	n.a.	N: ++ / ± (10-75%), C: ± / 0	N: ± / 0, C: 0
4C	8.5	+ / ±	n.a.	n.a.	n.a.	n.a.	N: ++ / ± (20-80%), C: ± / 0	N: ± / 0, C: ±
5C	9.5	+ / ±	n.a.	n.a.	n.a.	n.a.	N: ++ / ± (60-100%), C: ± / 0	N: ± / 0 (30%), C: 0
6C	9.5	trace	n.a.	n.a.	n.a.	n.a.	N: ++ / ± (50-90%), C: ± / 0	N: ± / 0, C: 0
7C	11.0	±	n.a.	n.a.	n.a.	n.a.	N: ++ / ± (50-90%), C: ± / 0	N: ± / 0 (30%), C: 0
8C	11.0	0	n.a.	n.a.	n.a.	n.a.	N: ++ / + (90-100%), C: 0	N: + / ±, C: 0
9C	11.5	0	n.a.	n.a.	n.a.	n.a.	N: ++ / + (90-100%), C: 0	N: + / ± (40%), C: 0
10C	12.0	0	n.a.	n.a.	n.a.	n.a.	N: ++ / ± (90-100%), C: 0	N: + / ± (40%), C: 0
11C	13.0	trace	n.a.	n.a.	n.a.	n.a.	N: ++ / + (90-100%), C: 0	N: + / ±, C: 0
12C	15.5	0	++	±	±	0	N: ++ / + (100%), C: 0	N: ++ / + (60%), C: 0
13C	16.5	0	++	+	±	±	N: ++ / + (100%), C: 0	N: +, C: ±
Adult normal testis		0	++ / +	++	+ / ±	++ / +	N: ++ / + (100%), C: 0	N: + (40%), C: ±
Remarks		In older patients heterogeneous	Spermatogonia containing tubuli more weakly stained especially in KS		Heterogeneous staining, strongest in central part		Weaker staining in tubules with Sertoli cells not fully differentiated	

\* Leydig cells (more or less differentiated) or interstitial cells. N, nucleus; ±, C, cytoplasm; n.a., not analyzed; (%), approx. % positive nuclei  
Score: ++, strong staining, +, intermediate staining, ±, weak staining, 0; no specific staining detected



**Figure 11.** Expression of AMH and inhibin B subunits in testicular biopsies of adolescent boys with KS. See Table 7 for details. **A**, AMH expression; strong uniform staining of all seminiferous tubules (patient 1KS). **B**, AMH expression; heterogeneous staining of SCO tubules (patient 6KS). **C**, inhibin  $\alpha$ -subunit expression; note the strong staining of Sertoli cells in SCO tubules and weaker staining in tubules with spermatogonia in the lower left (patient 9KS). **D**, inhibin  $\alpha$ -subunit expression; note the nuclear staining of some Sertoli cells (patient 10 KS, second biopsy). **E**, inhibin  $\beta$ B-subunit expression (patient 3KS). **F**, inhibin  $\beta$ B-subunit expression (patient 10 KS, second biopsy). Magnification x 200.





**Figure 12.** Expression of the androgen receptor in testicular biopsies of adolescent boys with KS and age-matched controls. See Table 7 for details. **A**, spermatogonia containing tubules identifiable at the right (patient 3KS). **B**, control 6C. **C**, a few spermatogonia-containing tubules identifiable in the center (patient 7KS). **D**, note nuclear staining of both Sertoli and Leydig cells (control 9C). **E**, AR predominantly in nuclei of Sertoli cells with only weak cytoplasmic staining, but note the abundant cytoplasmic expression in Leydig cells, with varying nuclear expression (patient 14KS). **F**, control 13C. Magnification x 200.

**Expression of inhibin B subunits.** Immunoexpression of the inhibin  $\alpha$ -subunit in Sertoli cells was relatively weaker in the older KS patients than in the younger ones (Table 7 and Figs. 11C and D). The majority of the KS samples (10 of 17), especially those from the older boys, showed clear nuclear staining (Fig. 11D); this nuclear expression was absent from all of the control samples. The Sertoli cells in tubules containing spermatogonia were less intensively stained than were those in the SCO tubules (Fig. 11C), a staining pattern of tubules also seen in controls, but one not as marked as in some of the KS samples. Expression of the inhibin  $\alpha$ -subunit also appeared in Leydig cells (Table 7 and Fig. 11D).

In contrast to the expression of the  $\alpha$ -subunit, expression of the inhibin  $\beta$ B-subunit expression in Sertoli cells remained stable in all KS specimens regardless of age (Table 7 and Figs. 11E and F).

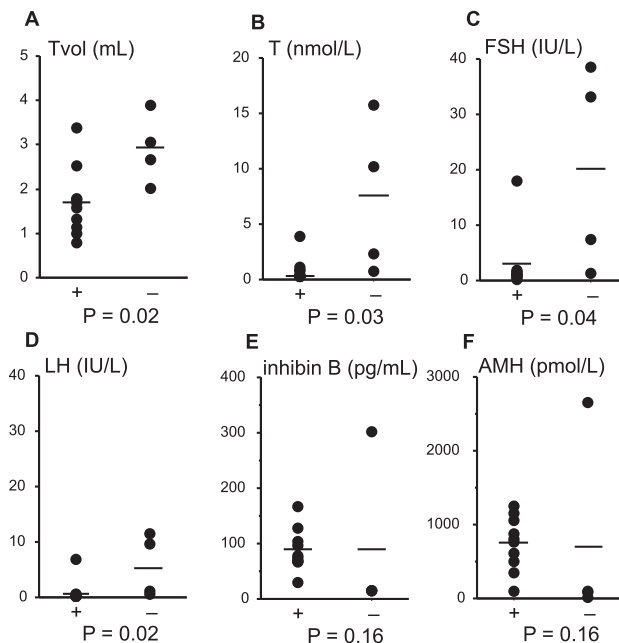
**Expression of the androgen receptor (AR).** Both in KS and controls the number of Sertoli cell nuclei expressing AR increased with age, but the proportion of stained nuclei was greater in the age-matched controls (Table 7 and Fig. 12). Expression was weaker in tubules containing not yet fully mature Sertoli cells, although no constant difference in intensity was evident in the KS samples between SCO tubules and seminiferous tubules containing germ cells (Figs. 12A and C). Weak cytoplasmic staining was detectable in Sertoli cells of all KS samples, but detectable in only the youngest control patients (age  $\leq 11.0$  years) (Table 7). Nuclear expression of AR in Leydig cells and interstitial precursor cells in the young patients in both groups was similar (Table 7 and Figs. 12A-D). In contrast to the controls, the older KS patients showed very strong AR expression both in the nuclei and cytoplasm of their Leydig cells (Table 7 and Figs. 12E and F).

## **Correlations between histological findings and serum hormone levels**

Correlations between serum reproductive hormone levels and findings of the testicular biopsy specimens were investigated to find whether hormone levels predict the presence or absence of germ cells. The 10 KS boys with spermatogonia detected with MAGE-A4 immunostaining of their first testicular biopsy had smaller testicular volumes ( $1.7 \pm 0.8$  vs.  $2.9 \pm 0.8$  mL,  $P = 0.02$ ), lower serum testosterone ( $0.9 \pm 1.1$  vs.  $7.2 \pm 7.0$  nmol/L,  $P = 0.03$ ), lower serum FSH ( $2.7 \pm 5.4$  vs.  $20.2 \pm 18.5$  IU/L,  $P = 0.04$ ), and lower serum LH ( $0.9 \pm 2.1$  vs.  $5.7 \pm 5.7$  IU/L,  $P = 0.02$ ) levels than did the four KS boys

with no spermatogonia present (Fig. 13). No differences between these two groups were detectable in serum inhibin B levels ( $87.8 \pm 38.9$  vs.  $87.0 \pm 142.7$  pg/mL,  $P = \text{ns}$ ) and AMH ( $746 \pm 365$  vs.  $713 \pm 1297$  pmol/L,  $P = \text{ns}$ ) at the time of the first testicular biopsy (Fig. 13). Patient 8KS constituted an exception; in the testicular biopsy specimens of all KS patients with measurable inhibin B levels ( $\geq 15.6$  pg/mL), germ cells were detectable, but in his testicular biopsy specimens, although obtained twice at an interval of 1.1 years, none were detectable. On both occasions his inhibin B (301 and 118 pg/mL) and AMH (2658 and 477 pmol/L) serum levels were normal. If subject 8KS is excluded from the analysis ( $n=13$ ), then inhibin B levels ( $87.8 \pm 38.9$  vs.  $< 15.6$  pg/mL,  $P = 0.01$ ) and AMH levels ( $746 \pm 365$  vs.  $64.3 \pm 42.5$  pmol/L,  $P = 0.01$ ) were predictive for the presence ( $n=10$ ) or absence ( $n=3$ ) of germ cells.

Serum AMH level decreased (Fig. 5B) concomitantly with its immunoexpression in the testes (Table 7 and Figs. 11A and B). Serum inhibin B levels decreased (Fig. 5A), but expression of both subunits remained detectable in the immunohistochemical analyses despite circulating inhibin B concentrations' being undetectable (Table 7, and Figs. 11 C-F).



**Figure 13.**

Testicular volume and serum hormone levels correlated with the presence of germ cells in testicular biopsies of 14 KS boys (mean age 11.9, range 10.1-14.0 year). +, germ cells detected,  $n = 10$ ; -, not detected,  $n = 4$ . Means for groups indicated by horizontal lines. Tvol = Testicular volume; T = testosterone.

## GENETIC MECHANISMS OF GONADAL FAILURE (III)

The impact on the phenotypes in Table 9 of all genetic features of the X chromosome listed in Table 8 was investigated. LH and testosterone levels clearly exceeding prepubertal levels were biochemical markers of onset of puberty.

**Table 8.** Genetic data of 14 nonmosaic 47,XXY boys

Patient	Origin of extra X chromosome	Number of CAG repeats	Inactivity ratio short: long allele	Preferentially active allele	Arithmetic mean of CAG repeats	X-weighted biallelic mean of CAG repeats
1KS	M I	17 : 17			17	
2KS	M I	19 : 22	69 : 31	long	20.5	21.1
3KS	M II	26 : 26			26	
4KS	M I	19 : 26	18 : 82 *	short	22.5	20.2
5KS	M II	22 : 22			22	
6KS	M II	22 : 22			22	
7KS	M I	22 : 24	69 : 31	long	23	23.4
8KS	P	20 : 21	17 : 83 *	short (P)	20.5	20.2
9KS	M II	26 : 26			26	
10KS	P	20 : 22	32 : 68	short (P)	21	20.6
11KS	M II	20 : 20			20	
12KS	P	24 : 24			24	
13KS	M I	22 : 22			22	
14KS	M I	20 : 21	50 : 50		20.5	20.5

M I = first maternal meiosis; M II = second maternal meiosis; P = paternal; \* = skewed X chromosome inactivation

**Parental origin of the supernumerary X chromosome.** The origin of the X chromosomes was unambiguously assigned for all cases (Table 8). Although the sample from his father was not available for patient 13KS, his supernumerary X chromosome was assigned as maternal, because all marker alleles of the proband were maternal. The extra X chromosome was paternal in 3 (21%) and maternal in 11 (79%) cases.

The parental origin of the supernumerary X chromosome did not influence the growth during childhood (ages 2-9 years). Relative heights in this period were  $0.63 \pm 1.03$  SD and  $-0.19 \pm 0.54$  SD for the 47,X<sup>m</sup>X<sup>m</sup>Y and 47,X<sup>m</sup>X<sup>p</sup>Y boys. Predicted adult heights were  $1.47 \pm 1.05$  SD and  $1.34 \pm 0.79$  SD for subjects with 47,X<sup>m</sup>X<sup>m</sup>Y and 47,X<sup>m</sup>X<sup>p</sup>Y, respectively. Furthermore, no difference existed in body composition

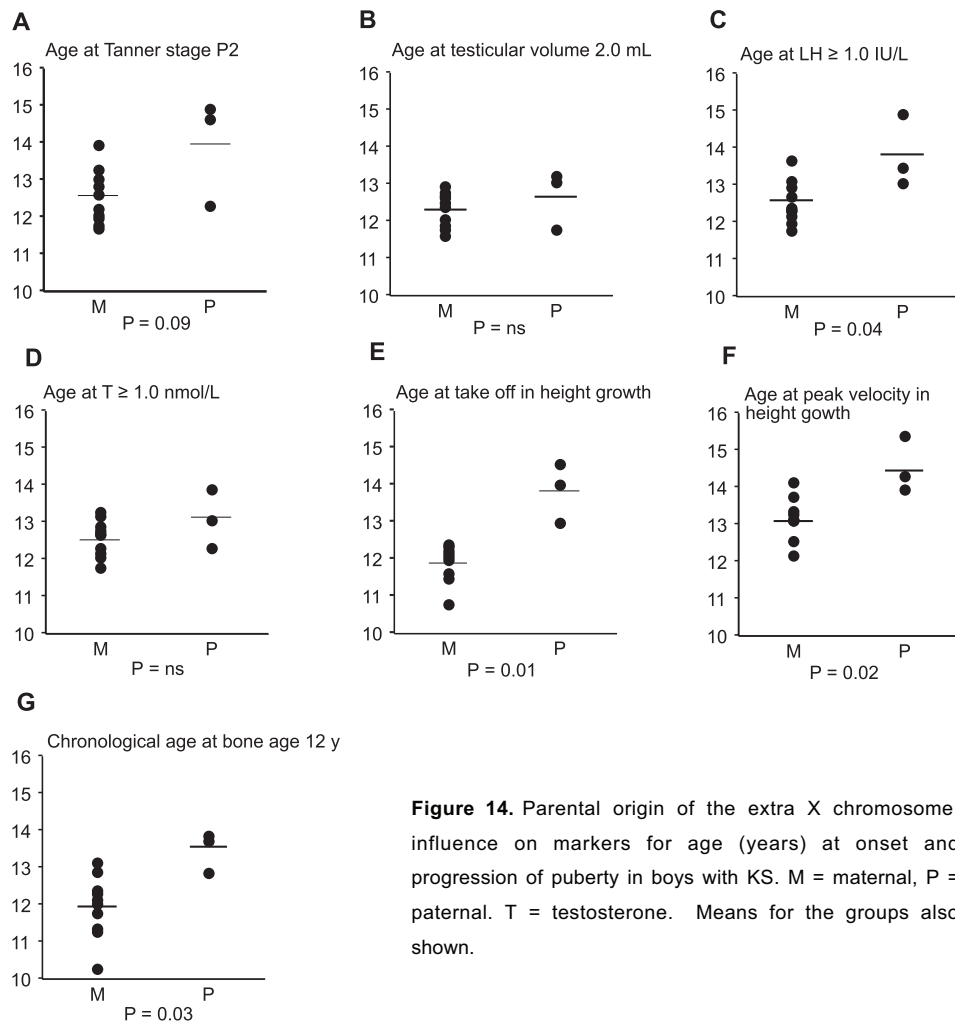
between these two groups during puberty (BMI after age 10: 47,X<sup>m</sup>X<sup>m</sup>Y, 19.6 ± 2.6 and 47,X<sup>m</sup>X<sup>p</sup>Y, 17.5 ± 0.8).

**Table 9.** Phenotypes investigated in 14 boys with KS

Growth	
	Mean height (SD) between ages 2 to 9 years, related to target height
	Predicted adult height (SD), related to target height
	Body composition during puberty (mean BMI after age 10 years)
Onset of puberty	
Clinical markers:	Age at Tanner stage P2
	Age at testicular volume 2.0 mL
Hormonal markers:	Age at serum LH ≥ 1.0 mIU/L
	Age at serum testosterone ≥ 1.0 nmol/mL
Progression of puberty	
	Age at acceleration of velocity (take-off) in height growth
	Age at peak velocity in height growth
	Chronological age at bone age (BA) 12.0 years
Testicular degeneration	
	Age at serum FSH ≥ 10.0 IU/L
	Age at serum LH ≥ 10.0 IU/L
	Age at serum inhibin B ≤ 32 pg/mL

The onset and progression of puberty was delayed in the 47,X<sup>m</sup>X<sup>p</sup>Y boys when compared to the 47,X<sup>m</sup>X<sup>m</sup>Y boys, as indicated by clinical markers and serum hormone measurements (Fig. 14). Tanner stage P2 was noted at age 12.5 ± 0.7 years in the 47,X<sup>m</sup>X<sup>m</sup>Y boys, and at 13.9 ± 1.4 years in the 47,X<sup>m</sup>X<sup>p</sup>Y boys ( $P = 0.09$ ) (Fig. 14A); the same trend was seen for the increase in testicular size ( $P = \text{ns}$ ) (Fig. 14B). There occurred a later increase in serum LH concentration in boys with a 47,X<sup>m</sup>X<sup>p</sup>Y karyotype; LH rose above 1.0 IU/L at age 12.5 ± 0.6 years in the 47,X<sup>m</sup>X<sup>m</sup>Y and at 13.8 ± 1.0 years in the 47,X<sup>m</sup>X<sup>p</sup>Y boys ( $P = 0.04$ ) (Fig. 14C). A similar trend appeared for testosterone ( $P = \text{ns}$ ) (Fig. 14D). As signs of a slower progression of puberty, the pubertal acceleration (take-off) in height velocity (47,X<sup>m</sup>X<sup>m</sup>Y 11.9 ± 0.5 years; 47,X<sup>m</sup>X<sup>p</sup>Y 13.8 ± 0.8 years;  $P = 0.01$ ) and peak velocity in height growth (47,X<sup>m</sup>X<sup>m</sup>Y 13.2 ± 0.6 years; 47,X<sup>m</sup>X<sup>p</sup>Y 14.5 ± 0.8 years;  $P = 0.02$ ) occurred later in the 47,X<sup>m</sup>X<sup>p</sup>Y subjects (Figs. 14E and F). The 47,X<sup>m</sup>X<sup>p</sup>Y boys were also older when they reached bone age 12 yr (47,X<sup>m</sup>X<sup>m</sup>Y 11.9 ± 0.8 years; 47,X<sup>m</sup>X<sup>p</sup>Y 13.5 ± 0.6 years;  $P = 0.03$ ) (Fig.14G). Since the pubertal increase in reproductive hormones is closely associated with acceleration

of the testicular degeneration process in KS subjects (I), the 47,X<sup>m</sup>X<sup>p</sup>Y boys had a later appearance of testicular degeneration.



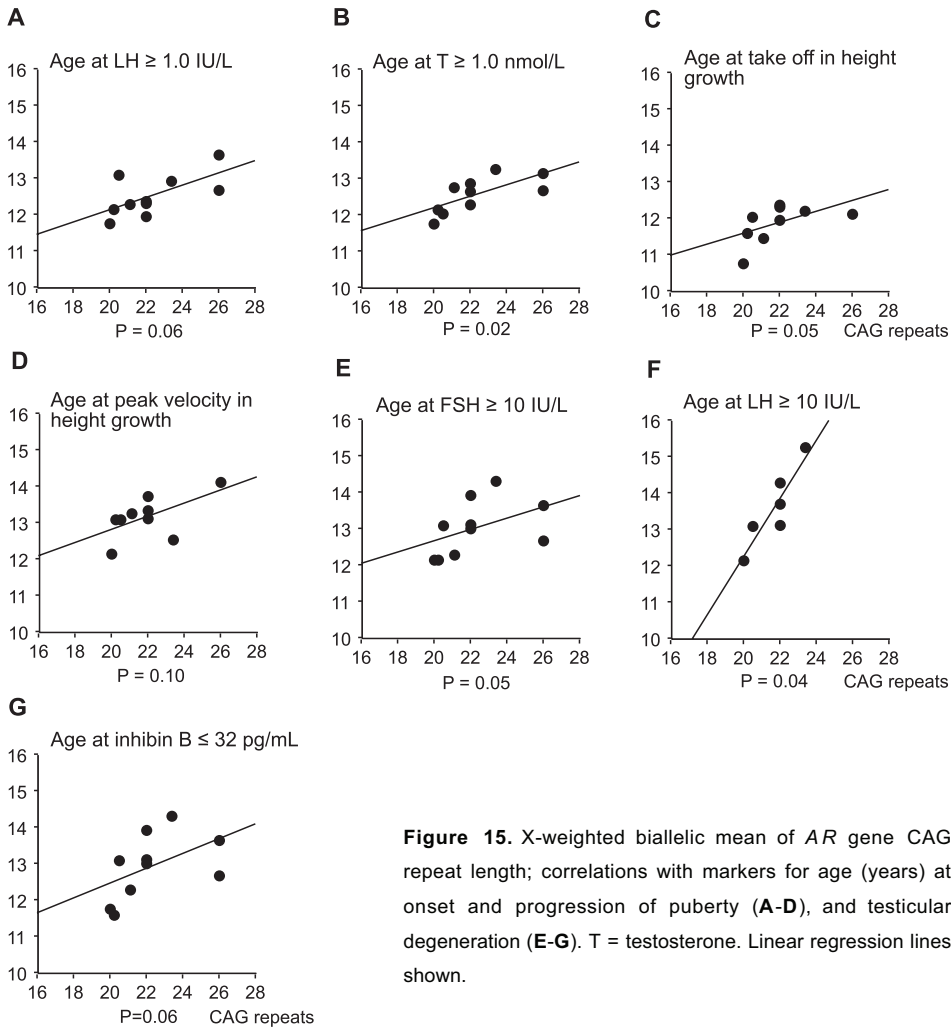
**Figure 14.** Parental origin of the extra X chromosome; influence on markers for age (years) at onset and progression of puberty in boys with KS. M = maternal, P = paternal. T = testosterone. Means for the groups also shown.

**X chromosome isodisomy / heterodisomy.** Judged by the microsatellite loci, 5 of the 11 boys with maternally derived extra X chromosome displayed uniparental X chromosome isodisomy (M II, Table 8). X chromosome isodisomy / heterodisomy did not influence the phenotypes listed in Table 9.

**X chromosome inactivation.** X inactivation status could be determined for 6 of the 14 subjects (43%), the remainder being homozygous for the *AR* gene CAG repeat (Table 8). Patients 4KS and 8KS showed skewed X inactivation, their ratios being 18:82 and

17:83 (Table 8). No significant differences in the phenotypic features listed in Table 9 were apparent between subjects with or without skewed X inactivation.

**CAG repeat length.** The lengths of the *AR* gene CAG repeat varied within the normal range, being from 17 to 26 (Table 8). No association was observable between the origin of the supernumerary X chromosome and the length of the CAG repeats (Table 8). Thus, for investigation of the influence of CAG repeat lengths on the phenotype, the 47,X<sup>m</sup>X<sup>p</sup>Y boys were excluded from these analyses, because of the major impact of the origin of that extra X chromosome. Spearman's rank correlations were calculated between CAG repeat lengths and the phenotypic features listed in Table 9. CAG repeat lengths did not influence the growth characteristics in Table 9.



**Figure 15.** X-weighted biallelic mean of *AR* gene CAG repeat length; correlations with markers for age (years) at onset and progression of puberty (A-D), and testicular degeneration (E-G). T = testosterone. Linear regression lines shown.

The CAG repeat length correlated with the hormonal markers for onset of puberty; boys with longer CAG repeats showed later increases in serum LH and testosterone levels (CAG length vs age at LH  $\geq 1.0$  IU/L;  $r_s = 0.63$ ,  $P = 0.06$ ,  $n = 10$ ; and CAG length vs age at testosterone  $\geq 1.0$  nmol/L;  $r_s = 0.78$ ,  $P = 0.02$ ,  $n = 10$ ) (Figs. 15A and B). No evident association appeared between CAG repeat length and the clinical markers for onset of puberty.

Progression of puberty as indicated by age at take-off ( $r_s = 0.70$ ,  $P = 0.05$ ,  $n = 9$ ) and age at peak velocity in height growth ( $r_s = 0.58$ ,  $P = 0.10$ ,  $n = 9$ ) tended to be slower in boys with a longer CAG repeat length (Figs. 15C and D). Furthermore, in those boys, testicular degeneration occurred later, as indicated by a slower increase in serum FSH and LH levels to hypergonadotropic levels over 10.0 IU/L (FSH;  $r_s = 0.65$ ,  $P = 0.05$ ,  $n = 10$ , and LH;  $r_s = 0.94$ ,  $P = 0.04$ ,  $n = 6$ ) (Figs. 15E and F), and a slower decrease in serum inhibin B levels below 32 pg/mL ( $r_s = 0.64$ ,  $P = 0.06$ ,  $n = 10$ ) (Fig. 15G).



## DISCUSSION

This prospective study comprised a longitudinal follow-up of growth, pubertal development, and serum reproductive hormone levels in 14 prepubertal and pubertal KS boys, aged 10.0 to 13.9 years at the start of the study. Each boy had a testicular biopsy specimen taken, which was cryopreserved for possible future use in assisted reproduction in adulthood. A small part of each specimen was thoroughly analyzed by histomorphometric and immunohistochemical methods. The impact of genetic features of the supernumerary X chromosome on growth, pubertal development, and testicular degeneration was also studied.

KS is the most common genetic form of male hypogonadism, but the complete clinical phenotype does not appear until after puberty and sometimes may never be fully expressed. The present study confirmed that during childhood and even during early puberty, pituitary-gonadal function in 47,XXY subjects is relatively normal. It showed that rate of pubertal progression in KS boys, as assessed by Tanner P and G stages, was normal. These results are in agreement with previous findings (Ratcliffe 1999; Salbenblatt et al. 1985; Sorensen 1992; Stewart et al. 1986; Topper et al. 1982). In addition, onset of puberty as judged by the appearance of pubic hair and initial testicular enlargement was normal. In the 47,XXY boys, skeletal maturation also fell within the normal range for healthy Finnish boys (Ojajärvi 1982). Thus, no phenotypic evidence was found for androgen deficiency in boys with KS during early and midpuberty.

Circulating serum testosterone levels in these KS boys did not differ from those in the control boys during early puberty, but from midpuberty onwards their testosterone levels were in the low-normal range, in agreement with the normal progression of puberty, and with data from others (Salbenblatt et al. 1985; Stewart et al. 1986; Topper et al. 1982). Only two studies on KS boys have demonstrated diminished testosterone secretion before puberty (Lahlou et al. 2004; Ross et al. 2005), but comprised only infant KS boys. However, circulating testosterone concentrations do not necessarily reflect androgen activity at tissue level. To address this question, serum levels of SHBG, PSA, and leptin were measured, the concentrations of which all are, at least to some extent, regulated by androgens. In the KS boys, serum SHBG decreased normally, and the levels did not differ from those observed in the controls. During puberty, serum PSA levels are in healthy boys a potential marker of testosterone activity. (Juul et al. 1997; Kim et al. 1999; Randell et al. 1996; Vieira et al. 1994). The

normal increase in serum PSA during puberty in the KS boys suggests sufficient androgen action in the prostate. Thus, as assessed by serum SHBG and PSA measurements, the KS boys displayed no evidence of diminished androgen action at cellular level.

A time- and dose-dependent decrease in leptin production occurs in human adipocytes exposed to testosterone (Wabitsch et al. 1997). During puberty, serum leptin levels decrease in close correlation with increasing testosterone levels (Ankarberg-Lindgren et al. 2001; Blum et al. 1997; Garcia-Mayor et al. 1997). Wabitsch et al. (1997) have shown that the leptin / BMI ratio is a good indicator of androgen action. The KS boys had higher leptin and higher leptin / BMI ratios than did control boys, suggesting diminished androgen action in adipose tissue. This does not, however, unequivocally reflect deficient androgen action, since serum leptin levels are also regulated by factors other than testosterone; e.g. one cannot exclude an impact of elevated E2 levels on serum leptin levels in KS boys.

No signs of hypergonadotropism were detectable in the KS boys during prepuberty or early puberty. After midpuberty, however, concomitantly with elevations in basal FSH and LH levels, their response to GnRH stimulation became abnormal. These observations are in agreement with earlier findings (Salbenblatt et al. 1985; Stewart et al. 1986; Topper et al. 1982) and suggest diminished testicular inhibition of gonadotropin secretion. In addition, after midpuberty the boys with KS developed low testosterone / LH ratios. Such changes in activity of the hypothalamic-pituitary-testicular axis thus probably represent a state of compensated hypergonadotropic hypogonadism attributable to the diminished responsiveness of Leydig cells to LH with advancing puberty.

INSL3, a new marker for Leydig cell function, has been suggested to be more sensitive than is testosterone to Leydig cell dysfunction and differentiation status (Bay et al. 2005; Bay et al. 2006; Foresta et al. 2004). The present study, like the cross-sectional one by Ferlin et al. (2006), showed a continuous increase in INSL3 levels during puberty in healthy boys, which provides an opportunity to use INSL3 as a Leydig cell-specific marker for the onset and progression of puberty. Furthermore, the dependence of INSL3 secretion on the differentiating action of LH was verified, because concomitantly with pubertal activation of LH secretion, INSL3 concentrations began to rise, and those boys treated with the aromatase inhibitor Lz who developed hypergonadotropic hyperandrogenism (Hero et al. 2005) had significantly higher INSL3 levels than did PI-treated boys. It was also clear that the positive relationship between LH and INSL3 was sustained in healthy boys throughout puberty; neither the present study nor previous studies have shown any negative feedback regulation by INSL3 on

LH secretion although the gene for the INSL3 receptor LGR8 is expressed in the pituitary (Bay et al. 2005; Bay et al. 2006; Ferlin et al. 2006; Foresta et al. 2004; Hsu et al. 2002).

In the KS boys, after their initial normal rise in serum INSL3 concentrations at onset of puberty, this secretion did not increase in a similar fashion as in the healthy boys, despite KS boys' high LH concentrations. Thus, it seems that in KS boys, Leydig cell function remains during puberty within the low-normal range, as indicated by both INSL3 and testosterone levels. The impact of low INSL3 levels during puberty and adulthood is difficult to estimate, since the precise role of INSL3 in adult men is unknown. At least in theory, because of the antiapoptotic effect of INSL3 on germ cells (Kawamura et al. 2004), suppression of INSL3 secretion may to some extent reduce the number of germ cells and hence impair fertility. The finding of LGR8 expression in meiotic and particularly in postmeiotic germ cells, and in Leydig cells but not in Sertoli or peritubular cells, supports this hypothesis (Anand-Ivell et al. 2006b). However, probably not even high INSL3 levels can suppress the massive germ cell apoptosis occurring in KS subjects during puberty.

The histomorphometric analyses and even more accurately the immunohistochemical stainings with the spermatogonia marker MAGE-A4 revealed that in early adolescence as many as 10 of the 14 boys with KS had germ cells in their testes, but the depletion of these cells accelerated at the onset of puberty. This presence of germ cells during peripuberty contrasts with findings of Muller et al. (1995), in which no germ cells were detectable in KS boys beyond the age of 2 years. A probable explanation is that all the boys in Muller's study were cryptorchid, whereas none in our group had any history of testicular maldescent. Ad spermatogonia that develop postnatally from fetal spermatogonia under gonadotropin and testosterone stimulation are of fundamental importance for the development of male fertility (Hadziselimovic and Herzog 2001). In the boys with KS, the fact that the number of these cells was markedly reduced indicates even before puberty a severely impaired fertility potential.

Whether germ cell differentiation is delayed in the testes of KS boys, especially whether germ cells in peripubertal boys still exhibit the features of gonocytes, was studied by examination of the expression of AP-2 $\gamma$  and of OCT-3/4. These are markers for gonocytes and neoplastic germ cells, and are frequently present in intersex gonads in children (Hoei-Hansen et al. 2004; Looijenga et al. 2003; Rajpert-De Meyts et al. 2003; Rajpert-De Meyts et al. 2004). The germ cells found in testicular biopsies of our KS patients were negative for AP-2 $\gamma$  and OCT-3/4, but positive for CHK2, a transducer of DNA damage signals and a tumor suppressor physiologically abundant in its inactive

form in fetal germ cells and in adult spermatogonia (Bartkova et al. 2001). Germ cells in adolescent KS boys had thus differentiated from gonocytes into spermatogonia. This finding was supported by the high expression of MAGE-A4 and of NY-ESO-1, which appear at protein level in fetal germ cells in the second trimester of pregnancy and subsequently persist in spermatogonia and to some extent in primary spermatocytes, especially NY-ESO-1 (Aubry et al. 2001; Bartkova et al. 2001; Rajpert-De Meyts 2006; Satie et al. 2002). Taken together, these results indicate that in KS, germ cell differentiation is—at least partially—blocked at the spermatogonium or early primary spermatocyte stage, since no gonocytes or pachytene spermatocytes were detectable. It seems that in KS, spermatogonia have difficulty entering meiosis; instead they proceed to apoptosis, a process that evidently accelerates at puberty, when differentiation to meiosis normally starts.

An additional rationale for including MAGE-A4 and NY-ESO-1 was that these two cancer-testis antigens are located on the X chromosome. All spermatogonia in KS patients and in the controls strongly expressed MAGE-A4, but NY-ESO-1 was expressed in a more heterogenous manner, as seen previously (Aubry et al. 2001; Satie et al. 2002). Intensity of staining between KS and controls was identical. Some SCO tubules expressed MAGE-A4 unspecifically, probably due to leakage of antigens from degenerating apoptotic spermatogonia. Nor did expression of the Sertoli cell marker MIC2 between KS subjects and controls differ. MIC2 is a pseudoautosomal gene located on the X and Y chromosomes (Visfeldt et al. 1999). This identical expression intensity indicates no impact of a double dose of these X-linked genes; at this developmental stage one of the gene loci is probably inactivated.

It seems that the presence of germ cells in the testes of adolescent KS boys correlates with serum levels of FSH, LH, testosterone, inhibin B, and AMH, but the current series did reveal one exception to this rule: Since the focal nature of the testicular degeneration process was also demonstrated here, the biopsies in that one patient (8KS) probably missed areas with tubules containing germ cells.

During puberty the testes of KS boys grow (Salbenblatt et al. 1985; Topper et al. 1982); this was also observed here. In healthy boys, proliferation of germ cells is predominantly responsible for pubertal growth of the testes (Cortes et al. 1987; Muller and Skakkebaek 1983). Since no spermatogenesis was detected beyond the development of adult spermatogonia, and the number of these cells was low, pubertal testicular growth in the KS boys was due almost solely to the proliferation of Sertoli and interstitial cells. This might suggest that the immature Sertoli cells of KS boys are responding to the pubertal increase in FSH stimulation, as seen in boys with a normal karyotype. On the other hand, their immature Sertoli Sa and Sb cells were incapable of

transforming into the adult Sc cell type, and EM revealed degenerating Sertoli cells, a rare finding in the testes of healthy early pubertal boys. The concept of Sertoli cells in KS boys regressing after the initial phase of Sertoli cell proliferation is in agreement with the changes observed in their inhibin B levels.

Secretion of the Sertoli cell hormone inhibin B increases significantly at the onset of puberty in healthy boys, but thereafter serum levels remain relatively constant (Andersson et al. 1997; Raivio et al. 1998). Inhibin B concentrations are regulated by FSH, and during puberty an inverse relationship develops between FSH and inhibin B (Andersson et al. 1997; Raivio et al. 2000). Inhibin B is thought to reflect Sertoli cell function during prepuberty and to become germ cell-dependent during midpuberty (Andersson et al. 1998b). In the KS boys, serum inhibin B levels were normal during prepuberty and early puberty, but the activation of the pituitary-gonadal axis resulted in a rapid decrease to unmeasurable levels. A strong correlation appeared between an increasing testosterone level and suppression of inhibin B and also of the other Sertoli cell marker, AMH.

Next came the exploration of the relationship of serum concentrations of testicular hormones (inhibin B, AMH, and testosterone) to the immunohistochemical expression pattern. The inhibin  $\alpha$ -subunit is expressed at the protein level in Sertoli and Leydig cells both in fertile and infertile men, but data regarding the location of the  $\beta$ B-subunit are not as uniform, probably because of differences in tissue processing (Anderson et al. 1998; Andersson et al. 1998b; Bergh and Cajander 1990; Fujisawa et al. 2004; Marchetti et al. 2003; Toppari et al. 1998; Vliegen et al. 1993). Three studies found  $\beta$ B-subunit immunostaining in Sertoli cells even in SCO tubules (Anderson et al. 1998; Fujisawa et al. 2004; Vliegen et al. 1993), whereas two others (Andersson et al. 1998b; Marchetti et al. 2003) found no  $\beta$ B expression in the Sertoli cells of adult men. Andersson et al. (1998b), studying testicular biopsies of prepubertal boys, detected the  $\beta$ B in their Sertoli cells. In the present series, all the KS specimens as well as the controls showed  $\beta$ B-subunit expression to be confined to the Sertoli cells, and also a weak  $\beta$ B-immunostaining of the Sertoli cells in the morphologically normal testicular biopsy of one adult control. However, in agreement with all these studies, the  $\beta$ B-subunit was detected to a varying degree in Leydig cells.

Expression of the inhibin  $\alpha$ -subunit in the tubules that contained spermatogonia was weaker than in SCO tubules, as has been already shown (Bergh and Cajander 1990). For some reason though, this pattern was especially evident in the KS patients. Overall, the immunostaining of the  $\alpha$ -subunit was weaker in older than in younger KS patients, probably due to the accelerating degeneration of the Sertoli cells. A sign of

this degeneration process may also be the aberrant nuclear expression of the  $\alpha$ -subunit in the Sertoli cells. Even when serum inhibin B was unmeasurable, both subunits were expressed in the Sertoli cells, suggesting that in KS, inhibin B synthesis is altered.

At puberty, AMH expression in Sertoli cells is down-regulated concomitantly with the appearance of meiotic germ cells and with up-regulation of the androgen sensitivity of the Sertoli cell, both being features of the final maturation of the Sertoli cells (Rajpert-De Meyts et al. 1999; Rey et al. 1993; Sharpe et al. 2003). It has been proposed that the dramatic suppression of AMH production requires meiotic entry in spermatogenesis (Al-Attar et al. 1997). However, the current study confirmed that the absence of germ cells did not prevent down-regulation of AMH during puberty, as has been already shown (Rajpert-De Meyts et al. 1999). But the KS boys showed a delay in disappearance of AMH expression that may be explained in part by the lack of influence of meiotically dividing germ cells, as suggested Al-Attar et al. (1997), and in part by hypoandrogenism (Rey et al. 1993). Furthermore, the Sertoli cells did not differentiate uniformly, as shown by their heterogeneous expression pattern, a pattern especially pronounced in the older KS boys.

In normal individuals, AR expression first appears in Sertoli cell nuclei just before the onset of puberty but before final maturation of the Sertoli cells, concomitantly with rising concentrations of FSH and testosterone (Sharpe et al. 2003). Expression of AR in the adult human testis, in order from strongest to weakest, occurs in Sertoli cells, peritubular myoid cells, smooth muscle cells of the blood vessels, and finally in Leydig cells (Suarez-Quian et al. 1999). In a study of adult cryptorchid testes, focal absence of AR expression in Sertoli cells correlated with a lack of local spermatogenesis, and the intensity of AR staining in Sertoli cells diminished as a function of severity (Regadera et al. 2001). In another study of AR expression in the testes of subfertile men, no differences were apparent between subjects as related to severity of conditions (Van Rooijen et al. 1995).

A study of one KS patient has detected AR expression in the nuclei of Sertoli and peritubular cells, but in contrast to healthy men, his Leydig-cell AR expression was located in the cytoplasm (Kotula-Balak et al. 2004). In the XXY mouse, immunoexpression has been nearly absent from the Sertoli cells and localized to the cytoplasm of the Leydig cells (Lue et al. 2005). In the absence of androgens, AR is located in the cytoplasm (Gasc and Baulieu 1986). In the KS boys, AR expression in Sertoli-cell cytoplasm and the smaller proportion of Sertoli cell nuclei expressing AR may be attributable to their low-normal testosterone levels. In agreement with the earlier studies (Kotula-Balak et al. 2004; Lue et al. 2005), cytoplasmic staining of

Leydig cells appeared in the older KS boys, probably as a sign of impaired function of their hypertrophied Leydig cells, as also indicated by their high serum LH levels and the leveling-off in their serum testosterone and INSL3 levels. No difference appeared, however, between the KS boys and their controls in AR immunoexpression in Leydig cell nuclei, nor were any constant differences evident between tubules containing spermatogonia and SCO tubules, a finding demonstrated also in one study of cryptorchid men (Regadera et al. 2001).

The present study found that genetic features of the X chromosome influence the onset and progression of puberty and the process of testicular degeneration. However, because the patients in this study were diagnosed in childhood because of their speech delay and behavioral problems (13 of 14), the present study may include ascertainment bias; phenotypic features may differ between totally unselected cohorts of patients diagnosed prenatally or in patients diagnosed in adulthood because of infertility. Earlier studies have shown that the supernumerary X chromosome is paternal in 40 to 60% and maternal in 40 to 60% of KS cases (Iitsuka et al. 2001; Lanfranco et al. 2004; Simpson et al. 2003; Thomas and Hassold 2003), while in the present study the percentages were 21% paternal (n=3) and 79% maternal (n=11).

Here, the three subjects with a paternal additional X chromosome showed later onset and slower progression of puberty, but showed no differences in body composition. Other studies (Jacobs et al. 1988; Lorda-Sanchez et al. 1992; Zinn et al. 2005) have suggested that parental origin of the extra X chromosome has no evident effect on the phenotypes of KS males. In the first of these studies, this view was based on the finding of a similar proportion of maternally and paternally derived cases among subjects diagnosed either prenatally and as newborns, or in adulthood because of signs of hypogonadism; no phenotypes were studied (Jacobs et al. 1988). The second study found no significant differences in a number of clinical features, including clinical signs of hypogonadism and including psychosocial problems discovered by a questionnaire, between KS patients with a maternal vs. paternal extra X chromosome (Lorda-Sanchez et al. 1992). The third study found that the parental origin of the supernumerary X chromosome had no impact on anthropometric and physical findings (Zinn et al. 2005). In contrast, Stemkens et al. found in their study of 61 KS males aged 2 to 56 years ( $23.6 \pm 15.2$ ), many of whom were on androgen substitution therapy, increased body size and more motor impairment and speech / language problems in the paternal X group (Stemkens et al. 2006).

The conclusions of the current study were based not only on anthropological findings, but also on measurements of serum reproductive hormone levels. Zinn et al. (2005) measuring FSH, LH, testosterone, and E2 concentrations in their subjects aged

0.1 to 39 yr, found no differences in testicular function between the maternally and paternally derived cases. This and the other studies (Jacobs et al. 1988; Lorda-Sanchez et al. 1992; Stemkens et al. 2006) were, however, cross-sectional, whereas the present study was longitudinal, and in the present series the phenotypic differences became apparent during follow-up.

It has been suggested that X chromosome inactivation patterns, especially skewed X inactivation, influence KS phenotypes (Iitsuka et al. 2001), but to date no study has thoroughly evaluated the impact of skewed X inactivation. In the study by Zinn et al. (2005) two subjects showed skewed X inactivation, but no association with phenotypic features, nor did Stemkens et al. (2006) find any differences. This aspect was not evaluated in the study by Zitzmann et al. (2004), who had five individuals with skewed X inactivation in their cohort of 77 adult KS males. Similarly, in the current study no differences were evident between the two boys with skewed X inactivation and the other 12 boys.

Isodisomy may lead to a double dosage of some harmful genes which escape X-inactivation. The present study and the 2005 study by Zinn et al. found no impact on the KS phenotype, but Lorda-Sanchez et al. (1992) found some minor differences in the prevalence of some clinical features between KS subjects with X chromosomes originating in MI and MII; their small number of subjects did not allow any statistical analysis.

The *AR* gene located on the X chromosome may play a particular role in the differences in KS phenotype. However, only one study has found a clear impact of the CAG repeat length of the *AR* on the KS phenotype. Zitzmann et al. (2004) found a positive correlation with body height and presence of gynecomastia, but an inverse association with bone density, testicular volume, and response to androgen substitution, and even with social status (Zitzmann et al. 2004). In the study by Zinn et al. (2005) the only parameter investigated that was associated with CAG repeat length was penile length; the correlation was inverse. Stemkens et al. (2006) found, however, no clear pattern of correlation with physical features or developmental problems. In the present study, those KS boys with a longer CAG repeat showed a later onset and slower progression of puberty and a slower testicular degeneration process, findings in agreement with diminished *AR* response to androgens when the *AR* gene has a longer CAG repeat. This also suggests that androgens may play a role in initiating this degeneration process. The finding of lower *AR* activity with a slower testicular degeneration process supports this hypothesis.



## SUMMARY AND FUTURE PERSPECTIVES

This study confirmed that KS boys have sufficient testosterone levels to allow normal onset and progression of puberty. However, although serum testosterone levels remained within the low normal range, the results indicate development of a relative testosterone deficiency from midpuberty onwards. At tissue level, adequate androgen action was suggested by normal pubertal changes in serum SHBG and PSA. On the other hand, an increased incidence of gynecomastia, higher leptin levels, plateauing of INSL3 levels, and exaggerated responses to GnRH stimulation indicate a relative androgen deficiency. This is also in agreement with the results from the histomorphometric and immunohistochemical analyses: Leydig cell hyperplasia and fibrosis of the interstitium developed with age, and altered immunoexpression of AR indicated diminished androgen action.

During minipuberty KS infants have lower testosterone levels than do healthy infants (Lahlou et al. 2004; Ross et al. 2005), so one proposal is that KS subjects may benefit from testosterone supplementation during the first 2 to 3 months of life, although we still lack evaluation of the role of minipuberty as a predictor of testicular insufficiency in KS. In the present study, no marked androgen deficiency appeared in prepubertal and pubertal KS boys; consequently, androgen supplementation does not seem necessary before midpuberty. However, studies regarding the efficacy of early androgen substitution on the classical symptoms of KS, and on its somatic, neurodevelopmental, and psychosocial outcome, are necessary. These studies should be placebo-controlled to determine what role androgen deficiency plays in exacerbating the 47,XXY phenotype. All the characteristics of the KS phenotype cannot be ascribed to the relative hypogonadism; other factors such as the excess of X-chromosome genes probably also have some impact.

Successful TESE-ICSI for men with nonmosaic KS resulting in the delivery of healthy children was first reported in 1998 (Palermo et al. 1998). The same group seven years later reported a sperm retrieval rate of 72% per TESE attempt, and once sperm was obtained, ICSI resulted in pregnancy in 46% of cycles (Schiff et al. 2005). This high retrieval rate may be due to preoperative treatment; most of the patients received an aromatase inhibitor or a combination of hCG and aromatase inhibitor to enhance intratesticular production of testosterone. Furthermore, their observation that sperm retrieval rate appeared to be lower (20%) in men who previously received exogenous androgens (Schiff et al. 2005), may argue against the routine treatment of

KS males with testosterone. In fact, testosterone treatment causes a marked inhibition of spermatogonial maturation (McLachlan et al. 2002). The concern for maintenance of fertility potential in young KS men must be balanced with the potential benefits of testosterone replacement.

Cryopreservation of semen samples containing very low numbers of spermatozoa from KS boys in early puberty is possible and should be offered to appropriate patients before the start of testosterone supplementation. The current study, however, showed a marked acceleration of germ cell depletion at the onset of puberty, so the expected success rate is exceedingly low, especially when taking into account the ability of boys to provide semen samples during early puberty. Another option if the biopsy sample contains haploid germ cells would be TESE. The possible future use of cryopreserved testicular samples containing spermatogonia, but not more mature germ cells, for infertility treatments would require *in vitro* maturation of spermatogonia into mature spermatozoa or at least into late / elongated spermatids. Recent studies indicate that human testicular tissue can be cultured for at least up to 3 weeks without essential loss of spermatogonia (Larsen et al. 2002; Sousa et al. 2002). Early results also suggest that meiosis and spermatogenesis may resume under culture conditions, yielding normal spermatids with some fertilizing potential (Sousa et al. 2002). However, at present this option for fertility preservation in boys before spermarche remains entirely experimental.

Mechanisms leading to depletion of germ cells start early in life, resulting in a decreased fertility potential even before puberty. This study also showed by several means that the testicular degeneration process accelerates at the onset of puberty. Whether the defect in the 47,XXY testis is intrinsic to germ cells or is due to inability of the Sertoli cells to support normal germ-cell development is unknown. Furthermore, we do not know whether the Leydig cell failure is part of a response to germ cell depletion / Sertoli cell injury or whether specific characteristics are inherent in the failure that are intrinsic to Leydig cells. The current study indicates that androgens may play a role in initiating the degeneration of the seminiferous tubules, and that AR polymorphism may play a role in pathogenesis of gonadal failure. The molecular mechanisms behind the testicular degeneration process in KS have remained elusive and require further research.

Genetic features of the X chromosome appear to play a part in modulating KS phenotypes. The present study has shown that parental origin of the supernumerary X chromosome and the length of the CAG repeat of the AR gene influence pubertal development. Conclusions should, however, be drawn with caution because of the

small number of patients in this and previous studies. Further studies including a larger number of subjects are therefore needed to confirm these preliminary results

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